01997.044100.1 (NEW) PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: NEUHOLD et al.	)	Group Art Unit: 1632
Application No.: 09/717,450	)	Examiner: Wilson, Michael C.
Filed: November 20, 2000	)	Confirmation No.: 5417
For: TRANSGENIC ANIMAL MODEL FOR DEGENERATIVE DISEASES OF CARTILAGE	)	Customer No. 45743 (NEW)
	)	April 7, 2006

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Third Declaration of Dr. Lisa A. Neuhold Under 37 C.F.R. §1.132

Sir:

1. I, Lisa A. Neuhold, Ph.D., am a citizen of the United States of America, and am more than twenty-one years of age. I hereby declare as follows:

2 I presently hold the position of Program Director, Genetics & Proteomics, NIAAA, Bethesda, MD, and have held this position for about four years.

3. I am the first-named inventor of U.S. Patent Application No. 09/717,450 (hereinafter "the '450 application").

4. My qualifications as a scientist, and in particular in the field of transgenic animals and gene-targeting, are set forth in the copy of my curriculum vitae, which is attached as Exhibit A.

5. The purpose of this declaration is to address the enablement-based and written description-based rejections of the claims currently pending and under consideration in the '450 application; these rejections are recited in the most recent United States Patent Office Communication, dated November 7, 2005 (hereinafter "the instant Office Action").

6. I have reviewed and am familiar with the instant Office Action, as well as the currently pending claims of the '450 application.

7. In addition, I have re-reviewed and am familiar with the Second Declaration of Dr. Lisa A. Neuhold (hereinafter "my second declaration"), which was originally filed during prosecution of the parent 08/994,689 application on August 31, 2000, and filed during the prosecution of the '450 application on April 30, 2002. I am familiar with the art cited in my second declaration at Tab 2 (i.e., Dietz and Sandell (1996) *J. Biol. Chem.* 271:3311-16 (hereinafter "Deitz") (Exhibit B) and Bosserhoff et al. (1997) *Dev. Dyn.* 208:516-25 (hereinafter "Bosserhoff") (Exhibit C)).

8. From reviewing the instant Office Action, it is my understanding that the Examiner believes that the phrase "chondrocyte-specific promoter," as recited in the pending claims, is neither adequately described nor enabled in the '450 application for any

promoter other than the type II collagen promoter. Apparently, it is the Examiner's belief that the phrase "chondrocyte-specific promoter" means a promoter that directs the expression of a gene in non-joint tissues at a level of 10% or less, and that only the type II collagen promoter disclosed in the '450 application fits within the Examiner's definition of "chondrocyte-specific promoter."

9. It is my opinion that the mouse CD-RAP promoter, which is referred to in my second declaration (and discussed extensively in the papers disclosed therein at Tab 2), is a chondrocyte-specific promoter, and that one of skill in the art would immediately recognize that the *CD-RAP* gene contains a promoter that is chondrocyte-specific.

10. The *CD-RAP* gene is described in Bosserhoff, which states that *CD-RAP* is a "gene specific to chondrogenesis [that] ... provides a template for study of chondrocyte-specific gene expression." (at p. 520). Bosserhoff shows that *CD-RAP* is expressed solely in the cartilage of the embryonic mouse, and is expressed solely in the cartilage of live 3-day-old mice. (Id. at pp. 517-520). It is my opinion that upon reading Bosserhoff, one skilled in the art would conclude that the *CD-RAP* gene contains a chondrocyte-specific promoter, and, using the disclosure of the present application, would also conclude that the mouse CD-RAP promoter may be used to produce a transgenic rat as recited in the pending claims.

11. The promoter for the chondrocyte-specific mouse *CD-RAP* gene was isolated and known in the art prior to filing the '450 application (see Bosserhoff, *supra*

(Exhibit C); Xie et al. (1998) *J. Biol. Chem.* 273:5026-32 (Exhibit D) and Xie et al. (2000) *Matrix Biology* 19:501-09 (Exhibit E)).

12. It is my opinion that the mouse CD-RAP promoter is a chondroctye-specific promoter that may be employed to make a transgenic rat as set forth in the pending claims of the '450 application, and that one of skill in the art would immediately recognize from the teachings in the art, and based on the disclosure of the present invention, that the promoter for the mouse *CD-RAP* gene may be employed to make a transgenic rat as set forth in the pending claims of the '450 application.

13. It is my opinion that one could isolate or synthesize the promoter for the mouse *CD-RAP* gene by routine methods, such as polymerase chain reaction (PCR), restriction digest and cloning, or even chemical synthesis.

14. Regardless of the appropriateness of an attempt to equate "joint-specific expression" with "chondrocyte-specific promoter," as the Examiner apparently attempts, it is also my opinion that one skilled in the art would conclude that the CD-RAP promoter fits within the embodiment of "joint-specific expression" mentioned (as "typical") on page 15 of the '450 application, and discussed by the Examiner at pages 3-4 of the instant Office Action (i.e., less than 10% expression in non-joint tissues). Deitz (Exhibit B) states at page 3315 that *CD-RAP* is found in "the cartilaginous tissues, but from none of the other tissues that were tested," while Bosserhoff (Exhibit C) shows that *CD-RAP* is

expressed solely in the cartilage of the embryonic mouse, and is expressed solely in the cartilage of live 3-day-old mice. (Bosserhoff at pp. 517-20).

15. I further declare that all statements made herein are, to my knowledge, true, and that all such statements are based on information I believe to be true.

16. I further declare that these statements are made with the knowledge that willful false statements are punishable by fine or imprisonment or both, under Title 18, Section 1001, of the United States Code, and that such willful false statements may jeopardize the validity of the instant application and any patent issued thereupon.

Respectfully submitted,

4/7/06

Date

Lisa A. Neuhold, Ph.D.

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Lisa Ann Neuhold
SS#: 213-76-5978

4 Hearthstone Ct.
Potomac, Maryland 20854
Phone: (240) 453-9734

Phone: (301) 594-6228
E-mail: lneuhold@mail.nih.gov

Current Position

I am a Health Scientist Administrator accomplished in program development in the areas of genetics, genomics, and proteomics. I oversee research grants and contracts to support state-of-the-art technology development to foster basic research. I am responsible for determining direction of program operations as well as identifying new areas of opportunity, development of initiatives and setting priorities for program development. I am also the NIH representative to Trans-NIH committees that coordinate NIH wide research efforts in genomics.

Accomplishments

- I organized a symposium, titled "High-throughput Proteomics in Alcohol Research" to increase the awareness of the alcohol research community in the technology of Proteomics. This symposium was held in conjunction with the Annual Research Society on Alcoholism meeting on June 21, 2003 and was attended by more than 150 people. Research proceedings are published in the Alcoholism: Clinical and Experimental Research Journal (Neuhold et al., 2003). For this symposium I received an award for outstanding performance. I was invited to edit a review volume entitled "Proteomics in Neuroscience."
- In collaboration with members of the genetics group, I reviewed the NIAAA portfolio and developed scientific initiatives in the areas of genetic, genomics, and proteomics. My portfolio includes QTL mapping, genetically modified animals, gene expression studies, proteomics, RNA microarrays, and invertebrate genetic screening grants.
- I co-organized a workshop titled "Exploratory Conference on the use of Stem Cells in Alcohol Research" held in Bethesda, MD on December 7, 2001.
- I wrote the following PAs and RFAs
  - RFA-AA-06-002, "Identification of Alcohol Biomarker Signatures." Release Date: March 23, 2005.
  - RFA-AA-06-001, "Genomic, Proteomic, and Metabolomic Fingerprints as Alcohol Biomarkers (SBIR/STTR)." Release Date: March 23, 2005.
  - PA-03-162, "Finding Genes for Alcohol-Related Behaviors and Risk for Alcoholism." Release Date: Aug. 7, 2003.
  - PA-03-141, "Gene-Environment Interactions Influencing Alcohol-Related Phenotypes and Diseases." Release Date: June 19, 2003.
  - RFA: AA-03-003, "Small Business Initiative for Alcohol Proteomics." Release Date: Oct. 1, 2002.
  - RFA: AA-02-010, "Stem Cell Research for Alcohol-Related Disorders." Release Date: March 13, 2002
- I have written briefing reports and annual reports on the data of my grantees as well as concept papers. I participated in writing strategic plans, press releases, and congressional justifications.
- I have completed contract training and program training.

Pharmaceutical Experience

I have substantial training in the application of pharmacological tools for drug development. Experience in developing transgenic and knockout mouse models of arthritis. I developed the first mouse model of osteoarthritis which is being used in drug screens. This work lead to a publication, a patent, and W-AR (Wyeth-Ayerst Research) presented me with an exceptional achievement award. I was also an invited speaker to the Annual Inflammation Research Association Meeting (9/00) and a Gordon Research Conference on Matrix Metalloproteinases (8/99). Generous team player with high organizational skills.

Accomplishments

- Designed, developed, & supervised production of transgenic and gene targeted mice for use in target validation in drug development. This includes creating a mouse model of osteoarthritis.
- Characterized osteoarthritis lesions in a line of MMP-13 transgenic mice. This work is the first satisfactory mouse model of OA and is also novel in its utilization of a unique combination of technologies, *i.e.,* the tetracycline regulatable system and chondrocyte-specific expression of a constitutively active human MMP13 protein (in collaboration with the Inflammation Division; Neuhold et al., 2001).
- Adapted the yeast homologous recombination system to aid in the development of targeting constructs.
- Trained & supervised research assistants.
- Generated transgenic rats expressing the apoptotic regulators, Bak and Bcl-xL (in collaboration with the CNS Division).
- Designed a rescue strategy to generate an inducible TACE knockout in the embryonic lethal TACE homozygous knockout mouse (in collaboration with the Oncology Division & Immunex Corp.).
- Expressed the CMV 30 kD protease in the lenses of murine eyes to generate an in vivo screen for protease inhibitors (in collaboration with the Infectious Diseases Division).

Postdoc Experience

Characterized the role of bHLH (basic helix-loop-helix) transcription factors (Myo D, myogenin, E12, E47, and Id1) on muscle cell proliferation/differentiation (myogenesis). My work lead to a publication in Cell (Neuhold and Wold, 1993). I also was an invited speaker at a Keystone Symposia on Molecular and Cellular Biology (1993).

Graduate Experience

Identified the DNA binding elements, including the aromatic hydrocarbon (Ah) receptor cis-acting element, responsible for the activity of the cytochrome P_1450 promoter. This work lead to a publication in Molecular and Cellular Biology (Neuhold et al., 1989).

Positions

9/02-Present	Program Director for Genetics & Proteomics, GS14, NIAAA, NIH, Bethesda MD
8/01-9/02	Program Director for Neurogenetics, GS13, NIAAA, NIH, Bethesda, MD
8/97-8/01	Senior Research Scientist I, Molecular Genetics Division, Wyeth-Ayerst Research (W-AR; Genetics Institute), Andover, MA
8/94-8/97	Research Scientist III, Molecular Genetics Division, W-AR, Princeton, NJ

Education

7/90-7/94	Postdoctoral Fellow (Laboratory of Dr. Barbara Wold), Division of Biology, California Institute of Technology, Pasadena, California
1985-1990	Ph.D., Zoology (Molecular Biology), University of Maryland, College Park, Maryland. Graduate student with teaching fellowship, Laboratory of Developmental Pharmacology (Nebert; Chief), NICHD, NIH, Bethesda, MD
1977-1981	B.S., Zoology, University of Maryland, College Park, Maryland

Awards and Honors

2005	Accomplishment Recognition Award (NIAAA/NIH)
2003-2004	Invited to be the editor of the book "Proteomics in Neuroscience"
2003	Accomplishment Recognition Award (NIAAA/NIH)
1999	MMP-13 TACE Discovery Team Award (W-AR/AHP)
1998	Exceptional Achievement Award (W-AR/AHP)
11/91-7/94	NIH Fellowship: NIAMSD (Award number: AR08203-02)
7/90-7/91	Howard Hughes Fellowship

Patent

Neuhold, L.A., and Killar, L., (1997). Patent No.: US 6,613,958 B1. Transgenic Mouse Model for Degeneration of Type II Collagen in Joints.

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Abstracts

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Cloning of a Retinoic Acid-sensitive mRNA Expressed in Cartilage and during Chondrogenesis*

(Received for publication, June 7, 1995, and in revised form, November 10, 1995)

Uwe H. Dietz and Linda J. Sandell‡

From the Departments of Orthopaedics and Biochemistry, University of Washington, Seattle, Washington 98195 and the Veterans Administration Medical Center, Seattle, Washington 98108

Retinoic acid (RA) is known to play a role in various aspects of skeletal development *in vivo*, including morphogenesis, growth plate maturation, and apoptosis. In cell culture, RA treatment of chondrocytes suppresses the differentiated phenotype characterized by production of type II collagen and aggrecan. In an effort to discover molecules involved in regulation of the chondrocyte phenotype or related to developmental processes such as chondrogenesis, mRNAs from bovine chondrocytes cultured with and without RA were amplified by reverse transcription-polymerase chain reaction (PCR) and compared by differential display. PCR products whose expression was inhibited by RA treatment were cloned. One cDNA encodes a molecule we call cartilage-derived retinoic acid-sensitive protein (CD-RAP), and its properties are described here. The full-length bovine CD-RAP mRNA was cloned after amplification by the rapid amplification of cDNA ends procedure, and a part of the rat CD-RAP mRNA was amplified by reverse transcription-PCR using sequence-specific primers. The bovine CD-RAP mRNA contains an open reading frame of 130 amino acids. CD-RAP mRNA expression, as determined by Northern blot analysis and *in situ* hybridization, was present only in cartilage primordia and cartilage. The inhibition of CD-RAP mRNA expression by RA *in vitro* was time- and dose-dependent and was tested over concentrations from 10^{-8} to 10^{-6} M. Southern blot analysis of genomic DNA indicated that CD-RAP was encoded by a single copy gene and that no other genes were closely related. What appears to be the human homologue of CD-RAP was recently isolated and cloned from a melanoma cell line and shown to function as a growth inhibitory protein (Blesch, A., Boberhoff, A.-K., Apfel, R., Behl, C., Hessdoerfer, B., Schmitt, A., Jachimcza, P., Lottspeich, F., Buettner, R., and Bogdahn, U. (1994) *Cancer Res.* 54, 5695–5701). Neither CD-RAP nor this protein showed any homology to known proteins. We speculate that, *in vivo*, CD-RAP functions during cartilage development and maintenance.

Retinoic acid (RA)[1] is known to play a role in the growth and differentiation of the central nervous system, skin, and skeleton. Depending on the concentration, it can induce different processes. At high doses, RA has teratogenic effects on pattern formation, predominantly in the limb (1), craniofacial structures (2), and the central nervous system (3). During normal skeletal development, RA may participate in induction and morphogenesis of limbs (4) and in growth plate maturation (5). RA can repress chondrogenesis *in vitro* (6, 7) and induce osteogenesis in various cell lines (8). In terminally differentiated avian chondrocytes, RA can stimulate proliferation and maturation to osteoblast-like cells as well as matrix mineralization (9).

RA-treated chondrocytes have been used as a model system to investigate the mechanisms of chondrocyte "dedifferentiation," where expression of type II collagen is repressed (10, 11). In chick (10), rabbit (11), and bovine (12) chondrocytes, the α_1-chain of type I collagen is increased, and in chick, both type I and III collagens are increased (10, 13). Expression of other matrix molecules is also known to be altered by treatment of chondrocytes with RA, including proteoglycans (14), fibril-associated collagens (12), and matrix metalloproteinases (15). In culture, RA-treated chondrocytes rapidly change morphology from a rounded, polygonal cell shape to a very distinct, flattened spindle shape.

As an approach to better understanding the changes induced by RA, we examined chondrocyte mRNA by the techniques of reverse transcription-PCR and differential display. Bovine chondrocytes cultured in the presence or absence of RA were compared for differences in expression of mRNA. Products amplified from mRNA species that appeared to be down-regulated by RA were selected for further analysis. In this report, we describe a cDNA derived from a small mRNA, the protein product that we term cartilage-derived RA-sensitive protein (CD-RAP). The CD-RAP mRNA contains an open reading frame of 130 amino acids and was detected only in chondroprogenitor cells and chondrocytes. In cultured chondrocytes, expression of CD-RAP mRNA was inhibited by RA in a time- and dose-dependent manner. Insight into a potential function for this molecule in cartilage may come from studies of the human homologue, which was recently characterized in a melanoma cell line as a growth inhibitory protein (16).

EXPERIMENTAL PROCEDURES

Materials—General laboratory chemicals, RNase A, and RNase T1 were from Sigma. Dulbecco's modified Eagle's medium, restriction enzymes, and the random priming kit were from Life Technologies, Inc. pGem11Zf+, reverse transcriptase, and the poly(A) tract kit were from Promega. Hybond N paper was purchased from Amersham Corp. The RACE kit was from CLONTECH (Palo Alto, CA). Differential display PCR was performed using primers from Gene Hunter Co. (Brookline,

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‡ To whom correspondence should be addressed: Dept. of Orthopaedics, ORT 112, Veterans Administration Medical Center, 1660 S. Columbian Way, Seattle, WA 98108. Tel.: 206-764-2931; Fax: 206-764-2598; E-mail: lsandell@u.washington.edu.

[1] The abbreviations used are: RA, retinoic acid; PCR, polymerase chain reaction; CD-RAP, cartilage-derived retinoic acid-sensitive protein; RACE, rapid amplification of cDNA ends; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; bp, base pair(s).

MA). [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were from Amersham Corp., and ^{35}S-UTP (800 Ci/mmol) was from DuPont NEN. pBluescript II SK(+) cloning vector was from Stratagene (La Jolla, CA). Pronase was from Calbiochem. Collagenase (Class 2) was from Worthington. Fetal calf serum was from Hyclone Laboratories (Logan, UT). dNTPs were from Pharmacia Biotech Inc.

Chondrocyte Cell Culture—Chondrocytes were prepared from bovine articular cartilage as described by Kuettner *et al.* (17). The isolated cartilage was digested for 1 h in 0.7% Pronase in Dulbecco's modified Eagle's medium at 37 °C, followed by two washes with phosphate-buffered saline. The cartilage was then digested by incubation with 0.15% collagenase for 5 h at 37 °C in Dulbecco's modified Eagle's medium. The released chondrocytes were filtered through nylon mesh and harvested by centrifugation. The harvested cells were plated onto 100-mm cell culture dishes at a density of 1×10^5 cells/cm^2. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 μg/ml ascorbic acid, and 50 μg/ml gentamycin at 37 °C in a humidified atmosphere containing 5% CO_2. All-*trans*-RA was dissolved in 95% ethanol at a concentration of 1 mg/ml and added to the prewarmed medium, which was then added to the culture. After 4 days of culture, RA was added to the chondrocytes at a concentration of 3×10^{-8}, 3×10^{-7}, or 3×10^{-6} M for up to 6 days. Control cultures were treated with equivalent concentrations of ethanol vehicle. Medium was replaced every 2 days.

RNA Isolation—For isolation of total RNA from chondrocytes, the cells were harvested by centrifugation and dissolved in lysis buffer (4 M guanidinium SCN, 25 mM sodium citrate, 0.5% sodium sarcosyl, and 0.7% β-mercaptoethanol). For isolation of total RNA from different soft tissues, a 5-fold volume of lysis buffer was added to the tissue pieces, followed by homogenization of the tissues. Total cellular RNA was then isolated by CsCl centrifugation according to the method of Chirgwin *et al.* (18). Poly(A)$^+$ RNA was separated from total RNA with magnetic beads after annealing to a biotin-labeled oligo(dT) primer using the poly(A) tract kit. The reaction was performed according to the manufacturer's protocol.

Northern Blot Analysis—RNA was electrophoresed in an 1% agarose gel containing 6% formaldehyde and 1 × MOPS buffer (1 × MOPS buffer = 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA). Transfer of RNA to Hybond N nylon membranes was carried out by capillary blotting with 20 × SSC (1 × SSC= 0.15 M NaCl and 0.015 M sodium citrate). After transfer, the nucleic acids were cross-linked to the membrane in a Stratalinker (Stratagene). For analysis of RNAs, cDNA probes were labeled with [^{32}P]dCTP by random priming and hybridized in 50% formamide, 5 × SSC, 5 × Denhardt's solution (19), 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA at 42 °C for 16 h. After hybridization, filters were washed twice in 2 × SSC for 5 min at room temperature and twice in 2 × SSC and 0.1% SDS for 30 min each at 65 °C. The washed filters were exposed to Hyperfilm-MP x-ray films.

Differential Display PCR—Differential display PCR was performed using the RNAmap™ kit from Gene Hunter Co. following the manufacturer's instruction.

RACE—RACE was performed by using a commercially available kit (Clontech). The reactions were carried out according to the protocol given by the manufacturer. The CD-RAP mRNA was reverse-transcribed using an oligonucleotide with the sequence 5′-TAGACTGAGCTCACTGGCAG-3′ (primer 3; see Fig. 1) as a primer. PCR amplification of the full-length CD-RAP cDNA was performed with the oligonucleotides 5′-CGCGGATCCTCACTGGCAGTAGAAATCCCATA-3′ (primer 4; see Fig. 1) and 5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATTCGATAG-3′ (primer 5; see Fig. 1). *Bam*HI and *Eco*RI cloning sites are underlined.

DNA Sequencing—DNA sequencing was performed according to the dideoxy method (20) using a sequencing kit from U. S. Biochemical Corp. following the manufacturer's instructions or using the DyeDeoxy™ Terminator Cycle sequencing kit protocol with an Applied Biosystems Model 373 DNA sequencer. All sequencing was performed on both strands of DNA.

Amplification of Rat and Mouse Homologues—5 μg of total RNA isolated from rat fetal skeleton or 13.5-day mouse embryos were reverse-transcribed using 30 pmol of a specific primer with the sequence 5′-CGCGGATCCGAGCTCACTGGCAGTAGAAATCC-3′ (primer 6; see Fig. 1). The reaction conditions were 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM spermidine, 5 mM dithiothreitol, 0.5 mM dNTPs, and 5 units of reverse transcriptase. PCR amplification was performed with the primer used for the reverse transcription and an upstream primer with the sequence 5′-CGCGAATTCATGCCCAAGCTGGCTGACCGGAA-3′ (primer 7; see Fig. 1). The reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.2 mM dNTPs, 50 pmol of each primer, and 1 unit of *Taq* polymerase in a total volume of 100 μl. Amplification was performed through 30 cycles in a Perkin-Elmer thermocycler. Each cycle was for 30 s at 96 °C, 45 s at 50 °C, and 2 min at 72 °C. The amplified fragment was digested with the restriction endonucleases *Bam*HI and *Eco*RI. A recognition site for both of the enzymes was created during the synthesis of the upstream primer and the downstream primer, respectively. The digested fragment was cloned into pGem11Zf$^+$.

Primer Extension Analysis—For primer extension analysis, a 35-mer oligonucleotide, 5′-CGGACTCTGAGCAAGACAGTGGCTAAGGCAGGAAT-3′ (primer 8; see Fig. 1), was synthesized. The primer was end-labeled by phosphorylation with [γ-^{32}P]ATP using T4 polynucleotide kinase. 10^5 cpm of the DNA primer were hybridized with 1 μg of mRNA isolated from chondrocytes in 30 μl of hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl, and 80% formamide). The hybridization mixture was first heated to 85 °C to denature the nucleic acids following incubation at 30 °C for 16 h. The nucleic acids were precipitated by addition of 170 μl of water and 400 μl of 100% ethanol at 0 °C for 1 h, collected by centrifugation, and washed with 70% ethanol. For reverse transcription, the primer/RNA hybrids were redissolved in 20 μl of reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 1 unit/μl RNasin, 50 μg/ml actinomycin D, and 5 units of avian myeloblastosis virus reverse transcriptase). The reaction was incubated for 2 h at 42 °C. The mRNA template was then removed by digestion with RNase A (5 μg/ml) for 30 min at 37 °C. After extraction with phenol/chloroform, the nucleic acids were precipitated by centrifugation and redissolved in 10 μl of formamide loading dye (80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol FF, and 1 mg/ml bromphenol blue). Half of the reaction was heated for 5 min at 95 °C and analyzed by electrophoresis through a 6% sequencing gel. The extended cDNA products were detected by autoradiography.

Southern Blot Analysis—For Southern blot analysis, genomic DNA was digested with restriction endonucleases and electrophoresed through 0.7% agarose gel in 0.04 M Tris acetate and 0.001 M EDTA, pH 8.0. After electrophoresis, the nucleic acids were denatured by incubation of the gels in alkaline solution (0.5 M NaOH and 1.5 M NaCl) for 30 min, followed by neutralization for 30 min in 0.5 M Tris-HCl, pH 7.2, 1.5 M NaCl, and 0.001 M EDTA. The nucleic acids were blotted and hybridized as described above.

In Situ Hybridization—Mouse tissue at 13.5 days gestation was prepared for *in situ* hybridization as described by Sandell *et al.* (21). Tissue was hybridized with antisense riboprobes of CD-RAP and type IIA procollagen mRNA. CD-RAP antisense RNA was transcribed from a mouse cDNA fragment cloned into pGem1Zf(+), which is described above. The plasmid was linearized by restriction with *Eco*RI before *in vitro* transcription. For cloning of type IIA procollagen mRNA, cDNA from a segment spanning exons 1 and 2 was amplified via PCR. The fragment was designed with 5′-*Eco*RI and 3′-*Bam*HI restriction sites and inserted into the corresponding sites of pGem3Zf(+). The total length of the cDNA insertion was 266 bp. It contained 59 bp of exon 1 and 207 bp of exon 2. Antisense RNAs were transcribed *in vitro* using Sp6 RNA polymerase in the presence of ^{35}S-UTP. Hybridizations were carried out at 40 °C overnight with antisense riboprobes with a specific activity of 3–5 × 10^7 cpm/ml of tRNA hybridization solution (50% formamide, 20% dextran sulfate, 20 mM dithiothreitol, 1 mg/ml tRNA, 300 mM NaCl, 10 mM Tris-Cl, pH 7.4, 10 mM Na_2PO_4, pH 6.4, 5 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin). Slides were washed according to the procedure of Ausubel *et al.* (19), followed by exposure to Beta-Max autoradiographic film for 3 days and analysis with an MCID image analysis system.

RESULTS

Identification of New Chondrocyte Molecules by Differential Display—Chondrocytes were isolated from bovine articular cartilage and plated on tissue culture dishes at a density of 10^5 cells/cm^2. After 4 days of culture, all of the cells were adhered as monolayers. They expressed mRNAs characteristic for extracellular matrix molecules such as type II collagen, aggrecan, and link protein, which were used as markers for the normal chondrocyte phenotype (22). The cells were then treated with RA at a concentration of 3×10^{-6} M for 6 days to modulate the chondrocyte-specific gene expression. At this time, synthesis of cartilage-characteristic type II and XI collagens was not detectable (data not shown). Messenger RNA from these cells was

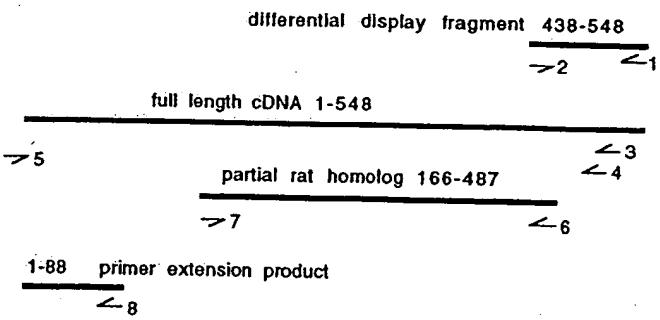

FIG. 1. **Diagram showing CD-RAP cDNA primers and products.** The sequences of the primers are shown under "Experimental Procedures."

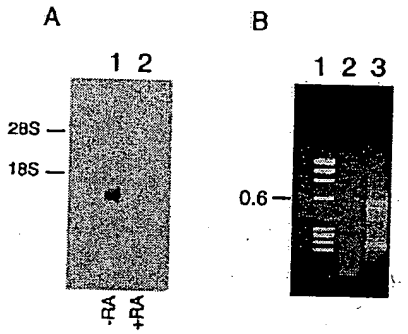

FIG. 2. *A*, detection of CD-RAP mRNA by Northern blot analysis. 5 μg of total RNA isolated from chondrocytes grown for 4 days in monolayer (*lane 1*) or treated for an additional 6 days with RA (*lane 2*) were electrophoresed through a 1% agarose gel and analyzed by Northern blot hybridization with a radiolabeled CD-RAP cDNA probe, followed by autoradiography. *B*, RACE of the full-length CD-RAP cDNA. mRNA from chondrocytes was reverse-transcribed with primer 3 (see Fig. 1). After ligation of the anchor primer to the 3′-end of the cDNA, PCR amplification was performed with primers 4 and 5 (see Fig. 1). An aliquot of the reaction was analyzed through a 2% agarose gel. *Lane 1*, molecular weight marker ϕX *Hae*III fragments; *lane 2*, PCR products after amplification of one-one hundredth of the anchor ligation reaction; *lane 3*, PCR products after amplification of one-tenth of the anchor ligation reaction. The size of DNA fragments is indicated in kilobases.

used for differential display by the method of Liang and Pardee (23). We isolated a total of 120 cDNA fragments that appeared only in the display of mRNA from untreated chondrocytes. Six of the 120 cDNA fragments were amplified from independent mRNAs that were down-regulated by RA. One of them was derived from an mRNA encoding a protein that was recently identified as melanoma growth inhibitory activity (EMBL accession number X75450). The expression of this molecule was detected only in a subset of melanoma cell lines, but not in any normal tissue (16). The sequences of the two primers that amplified the molecule for the original display were 5′-TTTTTTTTTTTTNG-3′ for the downstream PCR primer and 5′-GCAATCGATG-3′ for the upstream PCR primer (shown in Fig. 1). Reverse transcription-PCR using these primers amplified a cDNA fragment of 100 bp in length. Northern blot analysis confirmed that the mRNA, which was the original template for the reaction, could be down-regulated by RA *in vitro* (Fig. 2*A*). Because of the sensitivity of the expression of this mRNA to RA, it was called CD-RAP.

Full-length Cloning by RACE—From the results of the Northern blot hybridizations, it was determined that the full-length mRNA is a small molecule ~500 bases in length (Fig. 2*A*). RACE was used to amplify the full-length mRNA to its 5′-end (Fig. 2*B*). For RACE, we created a specific primer based on DNA sequence information derived from the 100-bp cDNA fragment we had cloned after differential display. This primer was used for reverse transcription of the CD-RAP mRNA. After synthesis of the cDNA, the mRNA template was hydrolyzed with alkaline solution, and the 3′-end of the cDNA, which represents the 5′-end of the mRNA, was ligated to an anchor oligonucleotide using T4 RNA ligase. A primer complementary to the anchor oligonucleotide and a nested downstream primer were used for PCR. The oligonucleotides used for reverse transcription and RACE are shown in Fig. 1. After 35 cycles in a thermocycler, an aliquot of the reaction was analyzed on a 2% agarose gel. Three distinct bands could be detected after agarose gel electrophoresis. Two of them migrated close together and were ~500 bp long. This coincided with the expected size of the full-length CD-RAP mRNA (Fig. 2*B*).

The cDNA fragments in the 500-bp range were purified from the agarose gel and cloned into pBluescript II SK(+). Nine individual clones were analyzed by dideoxy sequencing. All of them represented cDNA fragments amplified from the CD-RAP mRNA. The clone with the longest cDNA insert was used for further analysis. According to the sequence information from this clone, the full-length CD-RAP mRNA would be 548 bp long.

Primer Extension Analysis—The 5′-end of the CD-RAP mRNA was confirmed by primer extension analysis. A new primer with a length of 35 nucleotides was synthesized for the primer extension experiments (see Fig. 1). This primer started 88 nucleotides downstream of the sequence that appeared to be

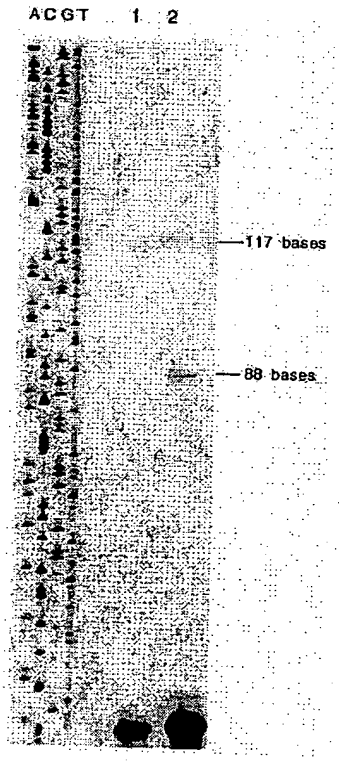

FIG. 3. **Definition of the 5′-end of the CD-RAP mRNA by primer extension analysis.** Primer 8 (see Fig. 1) was radiolabeled with [γ-^{32}P]ATP and used for reverse transcription of 1 μg of chondrocyte mRNA. The extended products were analyzed through a 6% sequencing gel and detected by autoradiography. In *lane 1*, the radiolabeled oligonucleotide alone was loaded. *Lane 2* shows the cDNA products extended from the radiolabeled primer. For the determination of the length of the synthesized cDNAs, a dideoxy sequencing reaction was loaded next to the primer extension reaction.

the 5′-end of the mRNA based upon the analysis of the RACE product. The primer was end-labeled with [γ-^{32}P]ATP and annealed to 1 μg of mRNA. The mRNA was then reverse-transcribed in the presence of actinomycin D to avoid self-priming. The products of the primer extension reaction were analyzed by electrophoresis through a 6% sequencing gel and detected by

```
human                                                                  ---gca-cc----gct-----c-
bovine   gtggggtttccttatgctactgcaagggagggcaggaggaagctggagaccccattcctgccttagccactgt          73

human    --------c------ac- --- --C C-- --- C-- --- -GC --T --- --- A-- ATC --- ---
bovine   cttgctcagagtccgtg ATG GCT TGG TCC TTG GTG TTT CTC GGT GTC GTC --- TTG CTG          129
bovine                    Met Ala Trp Ser Leu Val Phe Leu Gly Val Val *** Leu Leu          13
human                           Arg                 Cys                 Ile Ile

human    --- --- ---- T-C --A --- G-- -T- A-- --T G-T --T --- --- --- --- --- --- ---
bovine   TCT GCC TTC CCA GGG CCT AGT GCC GGG GGC CGC CCC ATG CCC AAG CTG GCT GAC CGG      186
bovine   Ser Ala Phe Pro Gly Pro Ser Ala Gly Gly Arg Pro Met Pro Lys Leu Ala Asp Arg      32
human            Ser         Gly Val Arg     Gly

rat        - C-- --- --A --- --- --G --T --- --T --T --T --- --- A-- --- --- --- ---
human    --- C-- --- --G --C C-- --G --- --- --- --T --- --- A-- --- --- --- ---
bovine   AAG ATG TGT GCC GAT GAG GAA TGC AGC CAC CCC ATC TCC GTG GCT GTG GCC CTT CAG      243
bovine   Lys Met Cys Ala Asp Glu Glu Cys Ser His Pro Ile Ser Val Ala Val Ala Leu Gln      51
human        Leu             Gln                             Met
rat          Leu                                             Met

rat      --- --- --- --- --- --T --- --C --- --- --T --- T-- -G- --- --- --- --A ---
human    --- --- A-- --- --C --- --- --A --- C-- --- --T --- -G- --- --- --- --- ---
bovine   GAC TAC GTG GCC CCT GAC TGC CGT TTC TTG ACC ATA CAC CAG GGC CAA GTG GTG TAT      300
bovine   Asp Tyr Val Ala Pro Asp Cys Arg Phe Leu Thr Ile His Gln Gly Gln Val Val Tyr      70
human        Met                                             Arg
rat                                                          Arg

rat      G-- --- --- --- T-G --A --- --T --A --- --T --- --- --- --- --- --G --- ---
human    G-- --- --- --- --G --- --- --T --- --- --- --- --- --- --- --C --- --- ---
bovine   ATC TTC TCC AAG CTC AAG GGC CGA GGG CGG CTC TTC TGG GGA GGC AGT GTT CAG GGA      357
bovine   Ile Phe Ser Lys Leu Lys Gly Arg Gly Arg Leu Phe Trp Gly Gly Ser Val Gln Gly      89
human    Val
rat      Val

rat      --- --- --- --- --- CTG --A --C -AC --- --- --- --- --- --- --- --T --C --G
human    --- --- --- --- --T CTG --- --- --C --- --- --- --- --- --- --- --T --C --A
bovine   GAT TAC TAT GGA GAC GGA GCT GCT CGT CTG GGC TAT TTC CCC AGT AGC ATC GTA CGT      414
bovine   Asp Tyr Tyr Gly Asp Gly Ala Ala Arg Leu Gly Tyr Phe Pro Ser Ser Ile Val Arg      108
human                     Leu         His
rat                       Leu

rat      --G --- -T- --T --- --- --- -G- --- GT- --- A-- --- --- --- GA- --
human    --G --- --- --- --- --- --- -G- --- GT- --- --- --- --- --- -A- --- --- ---
bovine   GAA GAC CAG ACC CTG AAA CCT GCC AAA ACC GAT GTG AAG ACA GAT ATA TGG GAT TTC      471
bovine   Glu Asp Gln Thr Leu Lys Pro Ala Lys Thr Asp Val Lys Thr Asp Ile Trp Asp Phe      127
human                           Gly     Val                     Lys
rat              Leu            Gly     Val     Met             Glu

human    --- --- --- ---------c---ccgctggccctgcc
bovine   TAC TGC CAG tgagctcagtctatactgtcattgctgttgtttctccccgactttatgcaaatacatcagcca      543
bovine   Tyr Cys Gln                                                                    130

bovine   agtgc-poly A                                                                   548
```

FIG. 4. **Full-length cDNA and protein sequences of bovine CD-RAP compared with the human and rat sequences.** The potential cleavage site of a hydrophobic signal sequence is indicated by the *arrow*.

autoradiography (Fig. 3). For determination of the length of the extended products, a dideoxy sequencing reaction was run next to the primer extension reaction. After autoradiography, two major extension products could be detected: a predominant band at 88 nucleotides and a minor band at 117 nucleotides (Fig. 3). The smaller band matched the size we expected for the full-length CD-RAP mRNA. The larger extended product indicates the potential presence of an alternative transcription start site, 29 bases farther upstream. Additional bands that yielded very weak signals were most likely premature termination products.

Fig. 4 shows the complete cDNA sequence of the CD-RAP clone. The bovine sequence spans the complete mRNA molecule from the 5′-start site of transcription to the poly(A) tail. A cDNA encoding a protein responsible for melanoma inhibitory activity (16) appears to be the human homologue of CD-RAP, and its sequence is shown in Fig. 4. No other homologous proteins were found in the data bank. The CD-RAP cDNA sequence revealed an open reading frame of 130 amino acids encoding a protein with a typical hydrophobic leader sequence followed by a unique domain protein. No linkage sites for *N*-linked carbohydrate (Asn-Gly-Ser/Thr) or *O*-linked carbohydrate (Ser-Gly) were found. The protein included four cysteine residues.

To screen for tissue specificity, a rat cDNA clone was prepared using the primers shown in Fig. 1. This cDNA probe was amplified by reverse transcription-PCR from total RNA isolated from skeletal cartilage of a fetal rat. The primers for reverse transcription and PCR were chosen from identical sequences in bovine and human CD-RAP cDNAs. Fig. 4 compares this 322-bp rat cDNA fragment with bovine sequence. The DNA conservation between these species is 85.5% for human and bovine, 90% for human and rat, and 87% for rat and bovine.

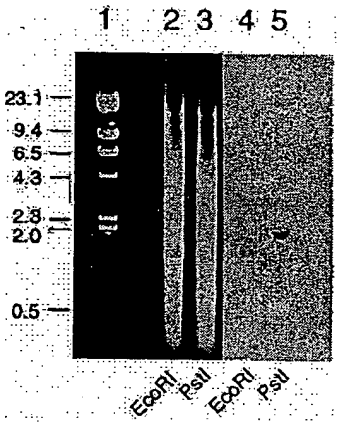

FIG. 5. **Southern blot analysis of CD-RAP.** 10 μg of bovine genomic DNA were digested with the restriction endonucleases *Eco*RI (*lane 2*) and *Pst*I (*lane 3*) and separated through a 0.7% agarose gel. The DNA fragments were blotted onto a nylon membrane and hybridized with a radiolabeled CD-RAP cDNA fragment. *Lanes* 4 and 5 show the hybridization products with the digested DNAs in *lanes* 2 and 3. *Lane* 1: molecular size marker (λ *Hin*dIII fragments). The size of DNA fragments is indicated in kilobases.

Southern Blot Analysis—Southern blot analysis was used to assess copy number of the CD-RAP gene and whether closely

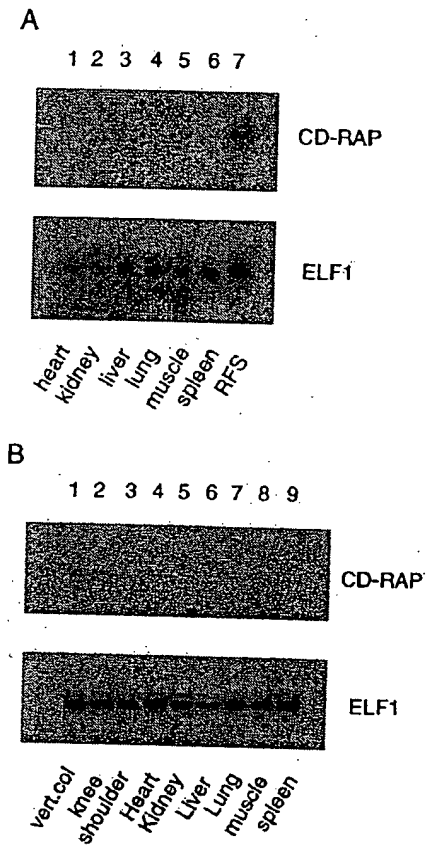

FIG. 6. **Tissue-specific expression of CD-RAP mRNA.** *A*, expression in adult rat tissues. 5 μg of total RNA from adult rat tissues (*lanes 1–6*) and rat fetal skeleton (*RFS*; *lane 7*) were used for Northern blot hybridization. Exposure time was 1 week. *B*, expression in fetal bovine tissues. 5 μg of total RNA from different cartilages derived from the vertebral column (*vert.col*; *lane 1*), knee (*lane 2*), and shoulder (*lane 3*) and from heart, kidney, liver, lung, skeletal muscle, and spleen (*lanes 4–9*, respectively) were analyzed by Northern blot hybridization with a radiolabeled CD-RAP cDNA probe. Exposure times were 1 week. As a control for equal loading of total RNA, the Northern blots shown in *A* and *B* were hybridized with a cDNA probe specific for ribosomal elongation factor 1 (*ELF1*) mRNA (22).

related genes could be found. 5 μg of bovine genomic DNA were digested with the restriction endonuclease *Eco*RI or *Pst*I. The digested DNAs were separated by electrophoresis through a 0.7% agarose gel and blotted onto nylon membranes. After hybridization and autoradiography, a single band was detected, indicating that CD-RAP is present as a unique gene (Fig. 5). When the blot was probed under low stringency conditions, no additional bands were observed, indicating that there are no other closely related genes in the genome. Similar results were obtained when the rat probe was tested on rat genomic DNA (data not shown).

Tissue-specific Expression of CD-RAP mRNA—To investigate the tissue distribution of CD-RAP mRNA, total RNAs from different rat tissues such as heart, lung, liver, kidney, skeletal muscle, and spleen were used for Northern blot hybridization. As a positive control, we included total RNA from skeletal cartilage of a fetal rat. After exposure to x-ray films for up to 2 weeks, a hybridization signal was detected only in total RNA from the rat fetal skeletal cartilage (Fig. 6*A*). To determine whether the absence of CD-RAP mRNA was related to a developmental stage, we tested for CD-RAP mRNA expression in a similar spectrum of fetal bovine tissues. In addition, RNA from different cartilages was analyzed, including the vertebral column, knee, and shoulder. Northern blot analysis showed the presence of CD-RAP mRNA in total RNA isolated from all of

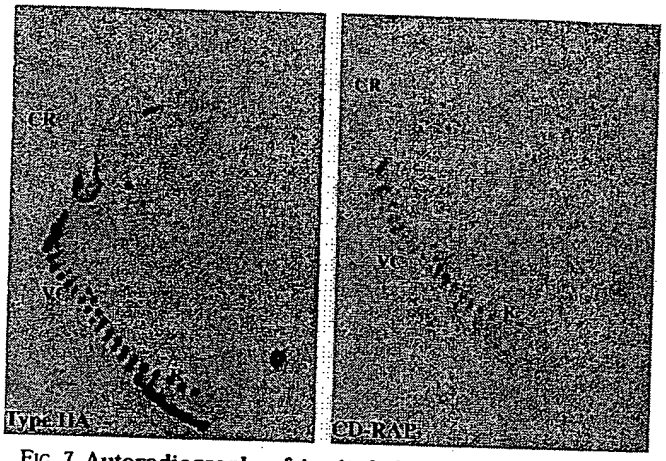

FIG. 7. **Autoradiographs of *in situ* hybridization with type IIA procollagen (*A*) and CD-RAP (*B*).** Tissue sections were from a 13.5-day mouse embryo. Tissues labeled are the primordia of the cranium (*CR*), vetebral column (*VC*), and ribs (*R*).

the cartilaginous tissues, but from none of the other tissues that were tested (Fig. 6*B*).

To confirm the tissue distribution of CD-RAP mRNA, *in situ* hybridization was performed in a mouse embryo. Sections of tissues from a 13.5-day embryo were used. A representative set of hybridizations is shown in Fig. 7. A probe for the mRNA of type IIA procollagen was used to localize cartilage primordia and to confirm the developmental stage of the mouse embryo. Comparison of the two hybridizations shows that CD-RAP mRNA is colocalized with the type II procollagen mRNA in the cartilage primordia of the developing vertebral column, ribs, cranium, and nasal septum. Interestingly, no expression of CD-RAP mRNA occurred in the non-cartilaginous tissues of the otic vesicle, where type II collagen expression is abundant.

Regulation of Gene Expression by RA—Since the concentration of RA originally used for the modulation of chondrocytes was much higher than physiological concentrations, we examined the effect on gene expression of lower RA concentrations (Fig. 8*A*). The effects of three different RA concentrations (3×10^{-6}, 3×10^{-7}, and 3×10^{-8} M) were tested. This represents a range from more physiological concentrations (3×10^{-8} M) to concentrations routinely used for dedifferentiation of chondrocytes *in vitro* (3×10^{-6} M). During culturing with RA, the morphology of the chondrocytes changed from a polygonal to a fibroblast-like cell shape as described previously (11). Total RNA was isolated after 2, 4, and 6 days of RA treatment. Under these conditions, CD-RAP mRNA expression showed dose- and time-related down-regulation by RA at all concentrations tested (Fig. 8*A*). In cells treated for 2, 4, and 6 days with the ethanol carrier only, expression of CD-RAP mRNA did not significantly change. The weaker signals after 4 and 6 days of ethanol treatment in Fig. 8*B* (*lanes 3* and *4*) are due to different amounts of total RNA loaded onto the gel. This is shown by the levels of elongation factor 1 mRNA detected after hybridization of the same blots with an elongation factor 1-specific cDNA probe (Fig. 8*B*, *ELF1*).

DISCUSSION

An mRNA encoding a cartilage protein, CD-RAP, has been cloned from cultured chondrocytes. It is down-regulated by RA *in vitro* in a time- and dose-dependent manner. The CD-RAP mRNA encodes a unique 130-amino acid protein containing a potential signal peptide, four cysteine residues, no obvious processing sites, and no sites of *N*- or *O*-linked glycosylation. We have characterized the full-length mRNA, demonstrated the start site of transcription, and showed that this gene is

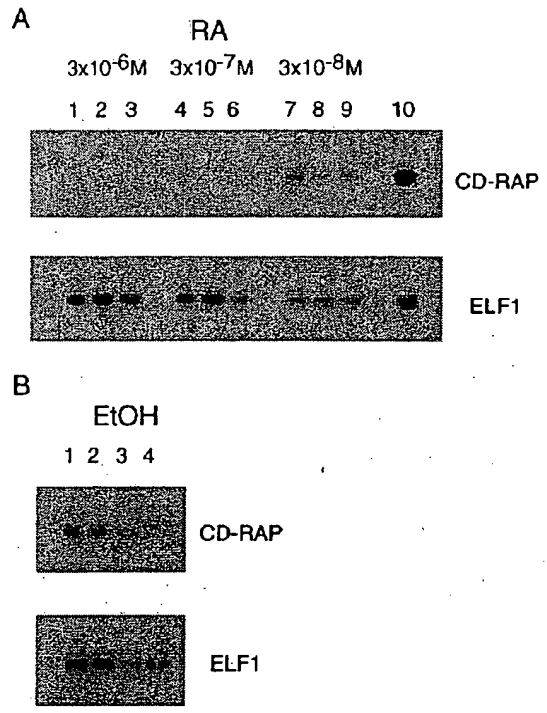

FIG. 8. **Time course and dose response of CD-RAP mRNA to RA.** *A*, total RNAs from chondrocytes treated with RA for 2, 4, and 6 days at a concentration of 3×10^{-6} M (*lanes 1–3*), 3×10^{-7} M (*lanes 4–6*), or 3×10^{-8} M (*lanes 7–9*) were analyzed. Total RNA from chondrocytes grown for 4 days in monolayer without RA was used to show CD-RAP mRNA levels before the RA treatment (*lane 10*). *B*, total RNA from chondrocytes grown for 4 days in monolayer (*lane 1*) and cultured for an additional 2, 4, and 6 days with the same amount of EtOH, which was used as the RA carrier (*lanes 2–4*), was analyzed for CD-RAP mRNA expression. In both *A* and *B*, 5 μg of each RNA were analyzed by Northern blot hybridization with a radiolabeled bovine CD-RAP cDNA probe. As a control for RNA loading, hybridization was performed with a cDNA probe for ribosomal elongation factor 1 (*ELF1*).

unique in the genome. *In vivo*, CD-RAP mRNA appears to be synthesized only by chondrocytes and chondroprogenitor cells. No expression was detected in RNA from various other adult rat or fetal bovine tissues; however, abundant expression was observed in rat fetal skeletal cartilage and in all bovine fetal cartilages tested. In embyonic tissue, CD-RAP mRNA is expressed in the cartilage primordia of 13.5-day mouse embryos. At this time, the cartilage primordia of the skeleton begins to form. Our results indicate that CD-RAP mRNA is synthesized by cells in the cartilage primordia and in differentiated cartilage. In the mouse embryo, expression of CD-RAP mRNA is similar to that of type II procollagen mRNA (21, 24). Current studies are underway to determine the temporal sequence of expression of type II procollagen splice forms and CD-RAP.

Previous studies have indicated that RA is potentially involved in cartilage differentiation. Although not itself a morphogen *in vivo*, RA is a capable of initiating pattern formation in chick limbs (25, 26) possibly through the induction of *hox* genes (27) in mesenchymal cells. RA can further stimulate the differentiation of chondrocytes to hypertrophic chondrocytes during growth plate maturation (28). In the growth plate, one role of RA may be to suppress the expression of typical chondrocyte extracellular matrix molecules such as type II collagen and aggrecan. RA-induced stimulation of type X collagen, alkaline phosphatase, and osteocalcin synthesis followed by mineralization and apoptosis may be important *in vivo* for development of the hypertrophic chondrocyte phenotype (29). The RA-dependent differences of CD-RAP mRNA levels *in vitro* may provide information relevant to understanding cartilage differentiation.

The cDNA we have characterized is almost certainly the bovine version of a molecule recently characterized in a melanoma cell line (16). Blesch *et al.* (16) showed that the expressed protein has growth inhibitory activity on certain melanoma cell lines. They found its expression limited to these melanoma cells and a neuroepithelial cell line. Addition of melanoma growth regulatory protein to the culture medium caused melanoma cells to round up and to decrease their incorporation of [^{3}H]thymidine. It is not clear how these activities could relate to chondrogenesis; however, a change in cell shape and decrease in proliferation are characteristics of the condensation phase of chondrogenesis. The relationship between the expression of CD-RAP in malignant cells and normal physiological expression by chondrosarcomas remains to be examined. Preliminary studies indicate the presence of CD-RAP mRNA in rat and human chondrosarcomas. Further studies will be needed to clarify the role of CD-RAP in the development and maintenance of cartilage.

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Mouse CD-RAP/MIA Gene: Structure, Chromosomal Localization, and Expression in Cartilage and Chondrosarcoma

ANJA K. BOSSERHOFF,[3] SEIJI KONDO,[1] MARKUS MOSER,[3] UWE H. DIETZ,[1] NEAL G. COPELAND,[4] DEBRA J. GILBERT,[4] NANCY A. JENKINS,[4] REINHARD BUETTNER,[3] AND LINDA J. SANDELL[1,2]*

[1]*Department of Orthopedics, University of Washington, VA Puget Sound Health Care Systems, Seattle, Washington*

[2]*Department of Biochemistry, University of Washington, VA Puget Sound Health Care Systems, Seattle, Washington*

[3]*Department of Pathology, University of Regensburg, 93053 Regensburg, Germany*

[4]*Mammalian Genetics Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland*

ABSTRACT **A cDNA encoding a novel protein has been previously isolated from two independent sources: melanoma cell cultures and chondrocytes. The protein from human melanoma cell lines and tumors is called melanoma inhibitory activity (MIA) (Blesch et al. [1994] Cancer Res. 54:5695–5701) and the protein from primary bovine chondrocytes and cartilaginous tissues is called cartilage-derived retinoic acid–sensitive protein (CD-RAP) (Dietz and Sandell [1996] J. Biol. Chem. 271:3311–3316). In order to investigate the gene regulation and function of CD-RAP/MIA, the mouse gene locus was isolated and analyzed. Developmental expression was determined by in situ hybridization to mouse embryos. Expression was limited to cartilaginous tissues and was initiated with the advent of chondrogenesis, remaining abundant throughout development. The mouse gene was isolated and sequenced from a 129Sv library and sequenced directly from an additional strain, B6C3Fe. The mouse CD-RAP/MIA gene is 1.5 kbp and consists of four exons. The promoter sequence of the gene contains many potential regulatory domains including 8 basic helix-loop-helix protein-binding domains and an AT-rich domain, both motifs shown to be present in the cartilage-specific enhancer of the type II procollagen gene. Other potential *cis*-acting motifs include binding sites for GATA-1, NF-IL6, PEA3, w-elements, NFκB, Zeste and Sp1. The gene, called *cdrap*, was localized to the end of an arm of chromosome 7 at the same site as the transforming growth factor β1 (Tgf-β1) and the glucose phosphate isomerase 1 (*Gpi1*) genes. Potential mouse mutants that mapped to the same region of chromosome 7 were identified. Two of the potential mutants with skeletal phenotypes were sequenced, pudgy (*pu*) and extra toes with spotting (Xs^J); however, no mutations were found in the coding sequence. To determine whether CD-RAP/MIA is associated with tumors of cartilage, mRNAs from a variety of rodent tissues and cell lines were screened. Expression was detected in a rodent tumor, the Swarm rat chondrosarcoma and a chondrosarcoma cell line derived from it, but not in other tissues or tumors of non-cartilage origin. Immunolocalization revealed CD-RAP/MIA protein localized in cartilage only. These results show that the normal expression of CD-RAP/MIA is limited to cartilage; however, pathologically, it is expressed both in melanoma and chondrosarcoma. The restricted expression of CD-RAP/MIA may provide an opportunity to monitor cartilage metabolic activity as well as the tumor activity of melanoma and chondrosarcoma.** ***Dev. Dyn. 208:516–525, 1997.***

Key words: MIA; melanoma; CD-RAP; chondrogenesis; mouse gene

INTRODUCTION

CD-RAP/MIA is a unique protein expressed normally by chondrocytes (Dietz and Sandell, 1996) and pathologically by melanoma (Blesch et al., 1994). mRNA encoding CD-RAP was independently cloned as a cartilage-derived retinoic acid–sensitive protein (CD-RAP) from bovine chondrocytes and as a melanoma inhibitory activity (MIA) from a human melanoma cell line. In melanoma cell lines, MIA functions to inhibit DNA synthesis and causes cell rounding (Blesch et al., 1994). MIA is expressed at high levels in malignant melanomas and at lower levels in some benign melanocytic nevi, but is not detected in normal melanocytes (van Groningen et al., 1995; Bosserhoff et al., 1996). CD-RAP/MIA DNA encodes a 130-amino acid secreted protein in bovine, rat, and mouse (Blesch et al., 1994; Dietz and Sandell, 1996) and a 131-amino acid protein in human (Blesch et al., 1994) with no identifiable functional domains except the signal peptide. CD-RAP expression is restricted to cartilage in the adult animal and is

*Correspondence to: Linda J. Sandell, Ph.D., University of Washington, Department of Orthopedics ORT 112, VA Puget Sound Health Care Systems, 1660 S. Columbian Way, Seattle, WA 98108.

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expressed during chondrogenesis in both the axial and peripheral skeleton (Dietz and Sandell, 1996). The normal physiological function of CD-RAP in chondrocytes is currently not known.

The human MIA gene has recently been cloned and analyzed (Bosserhoff et al., 1996). The gene is preferentially active in melanoma cells when compared to other cell lines and is inducible by the phorbol ester, phorbol 12-myristate 13-acetate. In order to study developmental regulation in situ and further explore the function of this new protein, the mouse gene was isolated and analyzed. Our combined investigation of the mouse gene demonstrates that MIA and CD-RAP are encoded by the same gene. Comparison of the human and mouse genes serves to focus attention on conserved potential regulatory domains. In addition to the physiological function of CD-RAP/MIA throughout chondrogenesis, we have explored the possibility that CD-RAP/MIA is expressed in cartilage tumors as well as melanoma.

RESULTS

Developmental Expression of CD-RAP/MIA

In situ hybridization was performed on mouse embryos from days 8.5 to 16.5 of gestation. No expression was observed at days 8.5 and 9.5. Some expression was seen at day 10 with stronger expression at day 11.5 particularly in cartilages of developing bone including the mandible and clavicle (Fig. 1A). Expression was correlated with the beginning of overt chondrogenesis, localized to differentiating chondroblasts and chondrocytes, and continued as mature cartilage formed. Expression was observed in the nasal process, basioccipital bone of the cranium, vertebral bodies, and pelvis by day 12.5 (Fig. 1B). Some expression was observed in the cartilaginous lung bronchi and the submandibular gland at day 14.5 (Fig. 1C). CD-RAP/MIA expression was not observed in the mesenchymal tissues destined to undergo chondrogenesis nor in other non-cartilaginous tissues such as somites, notochord, neuroepithelium, periosteum, perichondrium, and osteogenic cells of the mandible. Positive hybridization was observed in cartilages of both the axial and peripheral skeleton and in articular and nasal cartilage as well as cartilages destined to be replaced by bone. The vertebral bodies exhibit expression when cartilage was being formed in the centra (Fig. 1B,C) and subsequently only in the cartilaginous end plates of the vertebral bodies (Fig. 1C,D). No hybridization was observed in bone cells at anytime during development. Beginning at day 14.5 and continuing into day 15.5 (Fig. 1D), hybridization was observed in the cartilage of lung bronchi. The white dots resembling signals over the liver, kidney and myocardium result from blood cells but not from specific hybridization. This conclusion is based on two factors: 1) The white color was generated from elements much larger than the true white silver grains of the emulsion and these elements were not visible under brightfield microscopy, and 2) reverse transcription–polymerase chain reaction (RT-PCR) of isolated tissues did not reveal any mRNA for CD-RAP/MIA in the mouse or the human (Bosserhoff et al., 1996).

More detail can be observed at higher magnification (Fig. 1E,F). The cartilages of the vertebral arches (Fig. 1E) and lung bronchi (Fig. 1F) were readily observed.

Immunolocalization of CD-RAP/MIA in Cartilage

Polyclonal antibody was produced in rabbits against human recombinant MIA. Western blots and immunoprecipitation from melanoma cell lysates indicated that the antiserum was specific for CD-RAP/MIA. Clavicle cartilage is shown. Mouse embryos at day 13.5 were examined, and antiserum was used at a dilution of 1:400. Immunolocalization shows CD-RAP/MIA in the chondrocyte lacunae and reveals that CD-RAP/MIA is present in the cytoplasm of chondrocytes and in the immediately surrounding extracellular matrix (Fig. 2). Very little CD-RAP/MIA is detected in the interterritorial matrix between lacunae.

Structure of Mouse CD-RAP/MIA Gene

A diagram of the CD-RAP/MIA gene is shown in Figure 3. The structure is generally the same as the human gene with slight differences in the sizes of the introns and exons. A comparison between the murine gene and the human gene shows that the CD-RAP/MIA gene consists of four small exons interrupted by three introns. The intron–exon boundaries are in accordance with the consensus splice sites. The entire locus encoding the CD-RAP/MIA protein is encompassed within a small region of approximately 2.5 kb. The structure and size of the human and mouse genes are conserved, indicating a potential advantage in the compact nature of the gene. Figure 4 shows the entire sequence of the mouse gene: promoter, exons, and introns. The start site of transcription is estimated to be at −106 based on the 5′ end of two independently isolated mouse cDNA clones. The region 1386 bases upstream of the translation start site in the mouse gene was compared to the same region of the human gene. The first 550 bases upstream of the translation start site were similar to the human gene (68% homology), although there was some difference in the placement of the potential regulatory motifs. Both the human and mouse lack a TATA box close to the transcription start site, nor did they contain a CAAT box; however, both genes contain TATA sequences further upstream (−800 to −900 in the mouse, −590 to −730 in the human). The Sp1 site was conserved in both genes as is the case for many extracellular matrix genes (−108 in human, −106 in mouse). The most prominent feature of the promoter region of both genes was the presence of eight bHLH-binding motifs. Other motifs conserved between both the human and mouse genes in sequence and approximate location include binding sites for α-INF-2, C/EBP, c-Myb, GATA-1, GMCSF, NF-IL6, NKκB, PEA3, TCF-2, W-element, and ZESTE.

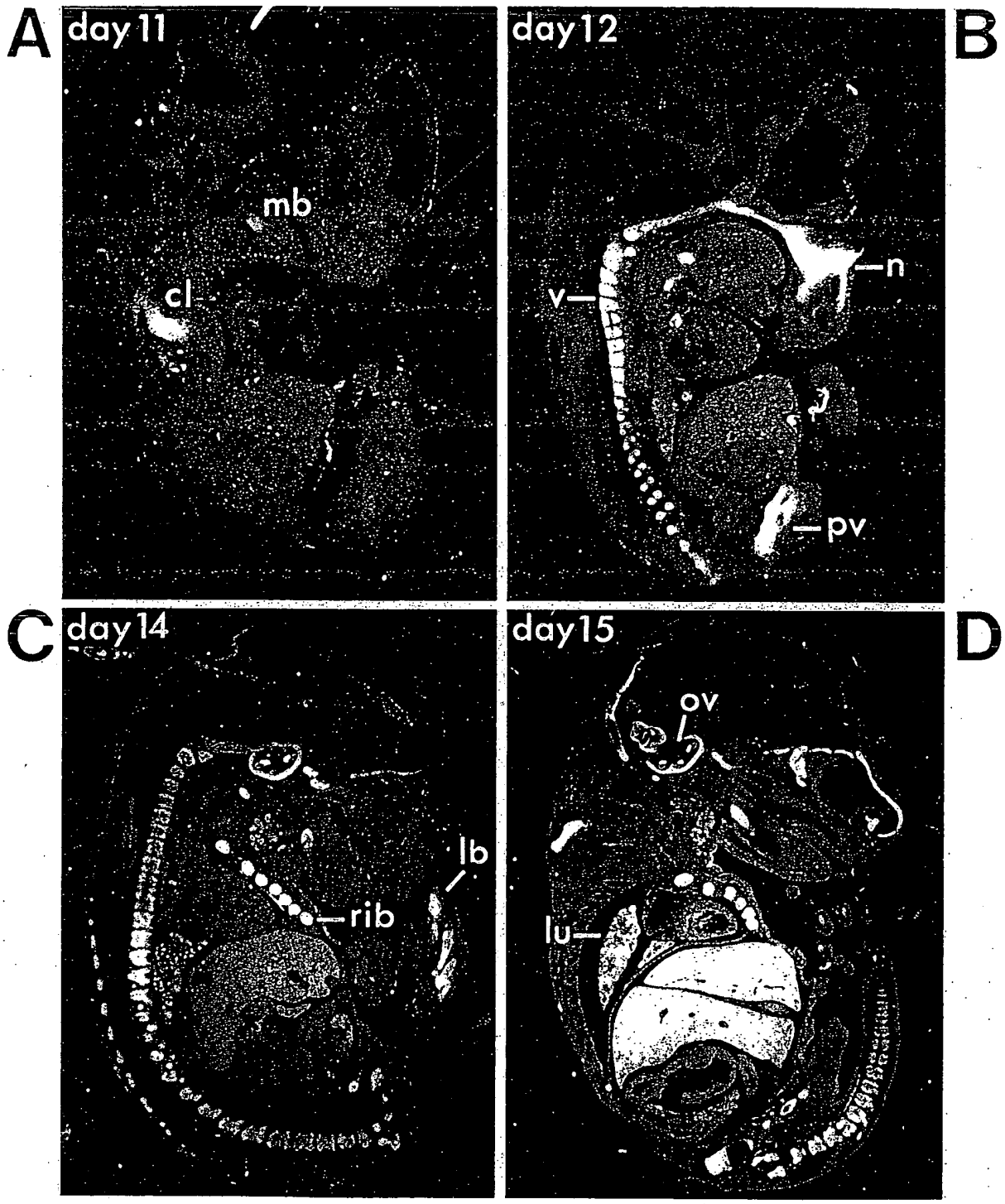

Fig. 1. In situ hybridization to mouse embryos. **A:** Day 11.5. **B:** Day 12.5. **C:** Day 14.5. **D:** Day 15.5. **E:** Higher magnification of day 13.5 vertebral arches and bronchi. **F:** Higher magnification of the limb bud at day 15.5. mb, mandible; c, clavicle; v, vertebral body; ov, otic vesicle; lu, lung; n, nasal process; pv, pelvis; lb, limb bud; va, vertebral arch; br, bronchi.

DNA sequence motifs previously shown to be involved in the expression of type II procollagen in cartilage were sought in the CD-RAP/MIA mouse genes. Two of the potential bHLH sites are a part of an extended homology between the CD-RAP/MIA gene and the type II collagen intron enhancer (indicated by double underlining in Fig. 4). An AT-rich sequence shown to be an important component of the cartilage-specific enhancer is present at −1036 in the mouse and −916 in the human CD-RAP MIA genes (Krebsbach et

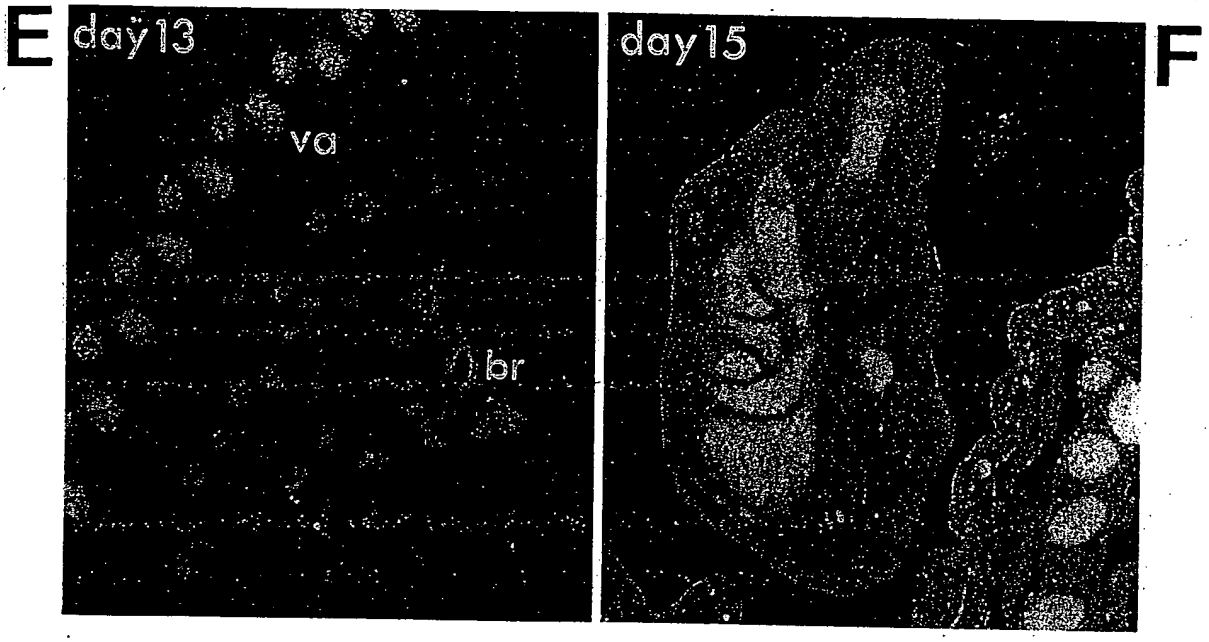

Figure 1. (Continued.)

al., 1996). Last, a pyrimidine-rich motif also included in the type II collagen gene enhancer (Mukhopadhyay et al., 1995) is present at −1367 in the mouse and −406 in the human gene.

Chromosomal Localization of the CD-RAP/MIA Gene

The mouse chromosomal location of *Cdrap* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J *Mus spretus*)F_1 × C56BL/6J] mice (Fig. 5). This interspecific backcross mapping panel has been typed for over 2,000 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a rat cDNA probe. The 19.0 kb *Hin*cII *M. spretus* RFLP (see Experimental Procedures) was used to follow the segregation of the *Cdrap* locus in backcross mice. The mapping results indicated that *Cdrap* is located in the proximal region of mouse chromosome 7 linked to *Pvs*, *Tgf*β1 and *Gpi*1. Although 175 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5), up to 186 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombinant frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are as follows: centromere-Pvs-1/181-*Tgf*β1-0/178-*Cdrap*-8/186-*Gpi*1. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) ± the standard error]

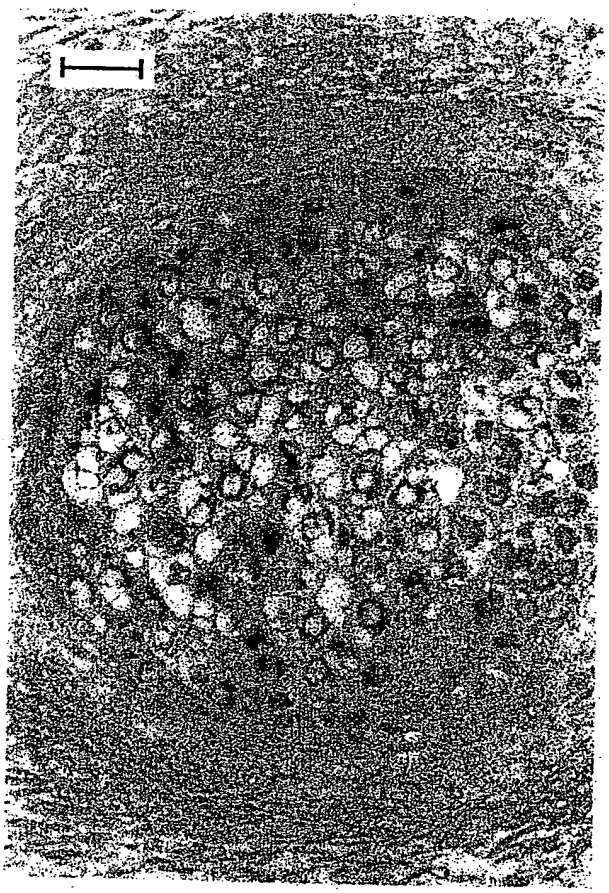

Fig. 2. Immunolocalization of CD-RAP/MIA. Tissue was prepared from a mouse embryo at day 13.5. Immunoreactivity is indicated by the red color product generally within lacunae of the chondrocytes. The tissue shown is the clavicle. Bar = 30 μm.

are-*Pvs*-0.6 ± 0.6-[Tgfβ1, Cdrap]-4.3 ± 1.5-*Gpi*1. No recombinants were detected between *Tgf*β1 and *Cdrap* in 178 animals typed in common, suggesting that the two loci are within 1.7 cM of each other (upper 95% confidence limit).

Expression of CD-RAP/MIA in Rodent Chondrosarcoma

In order to determine whether CD-RAP/MIA was expressed in cartilaginous tumors, RNA was isolated from three Swarm rat chondrosarcomas, a chondrosarcoma cell line derived from the Swarm tumor, a mouse mesangial cell line, a human leukemia cell line, HL-60, and mouse kidney. Positive hybridization was observed with RNA from the tumor tissue and the tumor cell line (Fig. 6). Mouse kidney, mesangial cell line, and the leukemia cell line RNAs were negative.

DISCUSSION

This article describes the isolation of the mouse gene encoding CD-RAP/MIA. While previously analyzed as separate genes, we now show that they are identical. The normal expression pattern of the gene was previously shown to be restricted to cartilage in a 13.5-day mouse embryo (Dietz and Sandell, 1996). Here we describe the developmental expression of this gene from days 11.5 through 16.5 of mouse embryogenesis and positive expression in a rodent chondrosarcoma. Expression of the gene was initiated at the beginning of chondrogenesis by differentiating chondroblasts. Expression continued in cartilage throughout the stages studied, including 3-day-old mice. Immunolocalization using a polyclonal antiserum shows that CD-RAP/MIA is present in chondrocytes; however, very little protein was visualized in the extracellular matrix. This pattern is also observed for other small, secreted proteins that have growth regulatory activity, such as BMP2 (Bostrom et al., 1995). The CD-RAP/MIA mRNA was originally isolated as a molecule co-expressed with type II procollagen, the predominant collagen of cartilage (Dietz and Sandell, 1996). Type II procollagen is synthesized in two alternatively spliced forms that are developmentally regulated (Ryan and Sandell, 1990; Sandell et al., 1991). As the chondroprogenitor or mesenchymal cells differentiate into chondroblasts, the splice form changes from the type IIA procollagen, characteristic of chondroprogenitor cells and non-cartilaginous cells, to the type IIB procollagen form characteristic of cartilage (Sandell et al., 1994; Nalin et al., 1995). CD-RAP/MIA expression was not found many of the places that the type IIA procollagen splice form was localized such as sclerotome of the somites, notochord, cranial mesenchyme, osteogenic cells of the mandible, kidney, periosteum, and perichondrium (Sandell et al., 1994). CD-RAP/MIA expression is therefore correlated with the expression of the chondrocyte-characteristic type IIB splice form of type II procollagen mRNA. The availability of a gene specific to chondrogenesis and co-expressed with type IIB procollagen provides a template for study of chondrocyte-specific gene expression.

The finding of expression of CD-RAP/MIA in three independent isolates of the Swarm rat chondrosarcoma and a cell line derived from a fourth isolate may be significant. This chondrosarcoma forms large tumors when implanted in normal rats (Choi et al., 1971) and the cell line shows many characteristics of mature chondrocytes (Mukhopadhyay et al., 1995). Preliminary results in human skeletal tumors indicate that CD-RAP is positive in all human chondrosarcomas as well (A. Howlett, H. Chansky, A. Bosserhoff, L.J. Sandell, unpublished data). The function of CD-RAP/MIA in this cartilaginous tumor may be similar to the function in the melanoma tumors or cartilage. Analysis of expression of CD-RAP/MIA may be used to monitor tumor occurrence or progression.

Comparison of the promoter between the mouse and human genes helps to identify DNA elements that are important for regulation. Of the sites in the human gene discussed by Bosserhoff and colleagues (Bosserhoff et al., 1996), an NFκB site was conserved although at a different location (−207 in the human and −819 in the mouse, both from the start site of translation), and the Sp1 site was conserved (−108 in the human, −106 in the mouse). Removal or mutation of the NFκB site from the human gene reduced activity of the gene construct in a melanoma cell line, B16; however, this site was not responsible for the stimulatory effect of the tumor promoter, PMA (Bosserhoff et al., 1996). In consideration of the potential of this gene for function during cartilage differentiation, a number of other conserved sites became interesting. The most outstanding characteristic of the promoter is the abundance of the bHLH motif (or E box), whose binding proteins function in determination of expression of differentiated cellular functions (Murre and Baltimore, 1992). bHLH-binding proteins are members of a large family that can form homodimers and heterodimers that activate and inhibit gene transcription. Two bHLH motifs have been identified in the chondrocyte-specific enhancer domain of the type II collagen gene (Wang et al., 1991; Mukhopadhyay et al., 1995) as well as the pyrimidine-rich domain (Mukhopadhyay et al., 1995). An AT-rich sequence motif was recently shown to be important for cartilage-specific expression of two cartilage genes: type II procollagen and link protein (Krebsbach et al., 1996). This motif was conserved between the mouse (−1036) and human (−916) CD-RAP/MIA at a similar location in the gene. Other sequences conserved between the mouse and human CD-RAP/MIA genes were also found in developmentally regulated genes: for example, Zeste is involved in control of segment polarity gene *wingless* (Manoukian et al., 1995) and the fibroblast growth factor receptor 3 gene (Perez-Castro et al., 1995), the gene responsible for multiple cartilage growth disorders. GATA-1 is generally considered specific to erythropoiesis and C/EBP, GMCSF, c-myb, and NF-IL6 are involved in differentiation of myeloid lineages (Zhang et al., 1996). These factors are involved in the choice of lineage by stimulating expression of specific growth factor receptors. An-

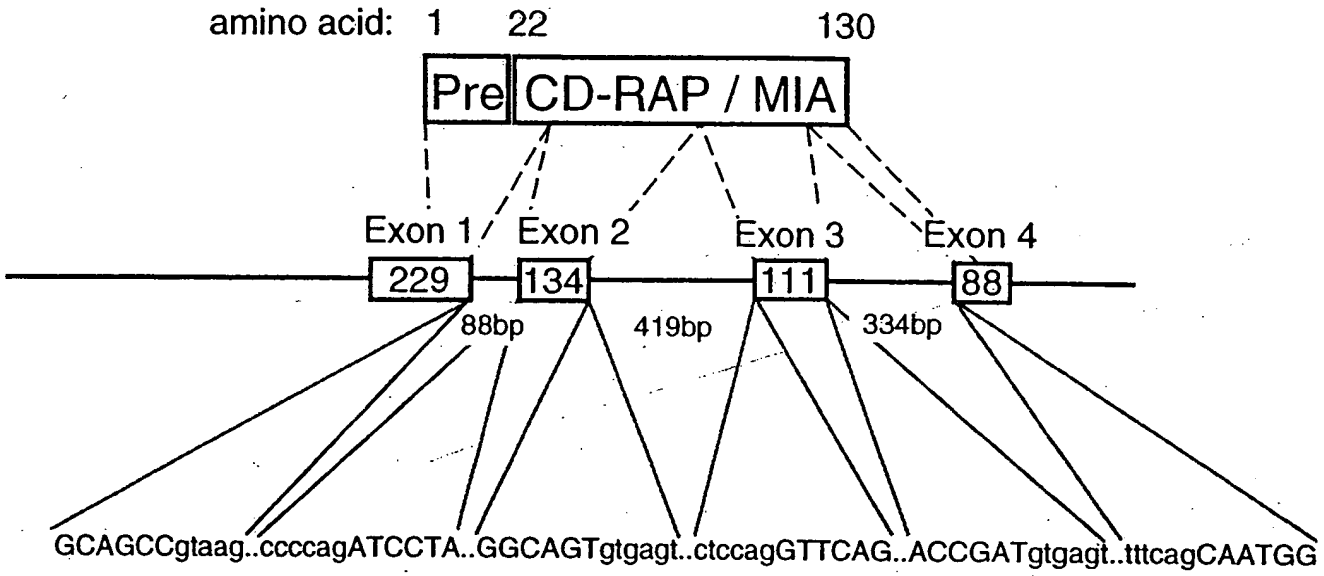

Fig. 3. Structure of the CD-RAP/MIA gene. Intron sequence appears in lower case, promoter and exons in upper case.

other group of potential *cis*-acting sequences present in the human and mouse CD-RAP/MIA genes are often found in immunological or oncogenic reactions, PEA3 (polyoma enhancer activator) (Monte et al., 1995), c-myb, ICSBP (interferon consensus sequence binding protein), and α-IFN (α-interferon) (Weisz et al., 1992).

Based on our expression studies of *Cdrap* shown here and previously (Dietz and Sandell, 1996), we would anticipate that a mutation in *Cdrap* would affect the development and/or maintenance of cartilage. To determine whether any mouse mutations affecting the skeleton were located in the vicinity of *Cdrap,* we compared our interspecific map of chromosome 7 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from Mouse Genome Database, a computerized database maintained at The Jackson Laboratory, Bar Harbor, ME). Three mutations were identified: a dominant mutation, extra-toes spotting (*Xs^J*), in which mice display a white belly spot and preaxial polydactyly of the forefeet; a recessive mutation, pudgy (*pu*), in which mice display a greatly shortened and highly irregular vertebral column and abnormal ribs and sternum; and another recessive mutation, claw paw (*clp*), in which mice show abnormally flexed joints as well as abnormal myelination (reviewed in Green, 1989). We have sequenced DNA from *Xs^J* and *pu* and have not found any mutations in the coding region (S. Kondo and L.J. Sandell, data not shown). The claw paw mutation has not been sequenced. An additional mutation called reduced pigmentation is also localized to the same position of chromosome 7; however, there is no evidence for CD-RAP/MIA expression in normal melanocytes (Bosserhoff et al., 1996).

The proximal region of mouse chromosome 7 shares a region of homology with human chromosome 19q (summarized in Fig. 5). In particular, *Tgf*β1 has been placed on human 19q13.2. The tight linkage between *Tgf*β1 and *Cdrap* in mouse suggests that *Cdrap* will reside on 19q13, as well. Indeed, the human gene was recently localized to 19q1.32-.33 (Koehler et al., 1996). With the availability of the mouse gene, future studies will address the function and regulation of expression of CD-RAP/MIA in genetically engineered mice.

EXPERIMENTAL PROCEDURES

In Situ Hybridization

In situ hybridization to paraffin-embedded mouse embryo sections was described previously (Moser et al., 1995). Briefly, slides were pretreated with proteinase K (1–10 μg/ml) for 30 min at 37°C fixed in 4% paraformaldehyde/PBS (pH 7.0) for 5 min, washed twice in H_2O, and acetylated in acetic anhydride diluted 1:400 in 0.1 M triethanolamine (pH 8) for 10 min at room temperature. Finally, slides were washed twice with H_2O, dehydrated in ethanol, air dried, and prehybridized for 4 hr at 50°C in 50% formamide, 10% dextran sulfate, 10 mM Tris pH 8, 10 mM NaPi pH 7. 2× SSC, 5 mM EDTA pH 8, 150 μg/ml tRNA, 10 mM DTT, 10 mM β-mercaptoethanol. Hybridizations were performed in the same mix supplemented with 5×10^4 cpm/μl of ^{35}S-labeled sense or antisense riboprobe at 50°C overnight. The slides were washed twice at 50°C in 50% formamide/2× SSC for 30 min and again twice in 2× SSC for 5 min. After RNase A treatment (20 μg/ml) for 30 min at 37°C, slides were washed again five times in 50% formamide/2× SSC at 65°C for 1 hr or overnight at 55°C, rinsed in 2× SSC, dehydrated, coated with Kodak NTB 2 emulsion, and exposed for 2 weeks.

Immunolocalization of CD-RAP/MIA

Antiserum was raised in rabbits against recombinant human MIA by Boehringer-Mannheim, Penzburg, Germany. Western blots and immunoprecipitation from melanoma cell lysates indicated that the antiserum was specific for CD-RAP/MIA. Thirteen and a half-day-old mouse tissue was used in this study. Immunohisto-

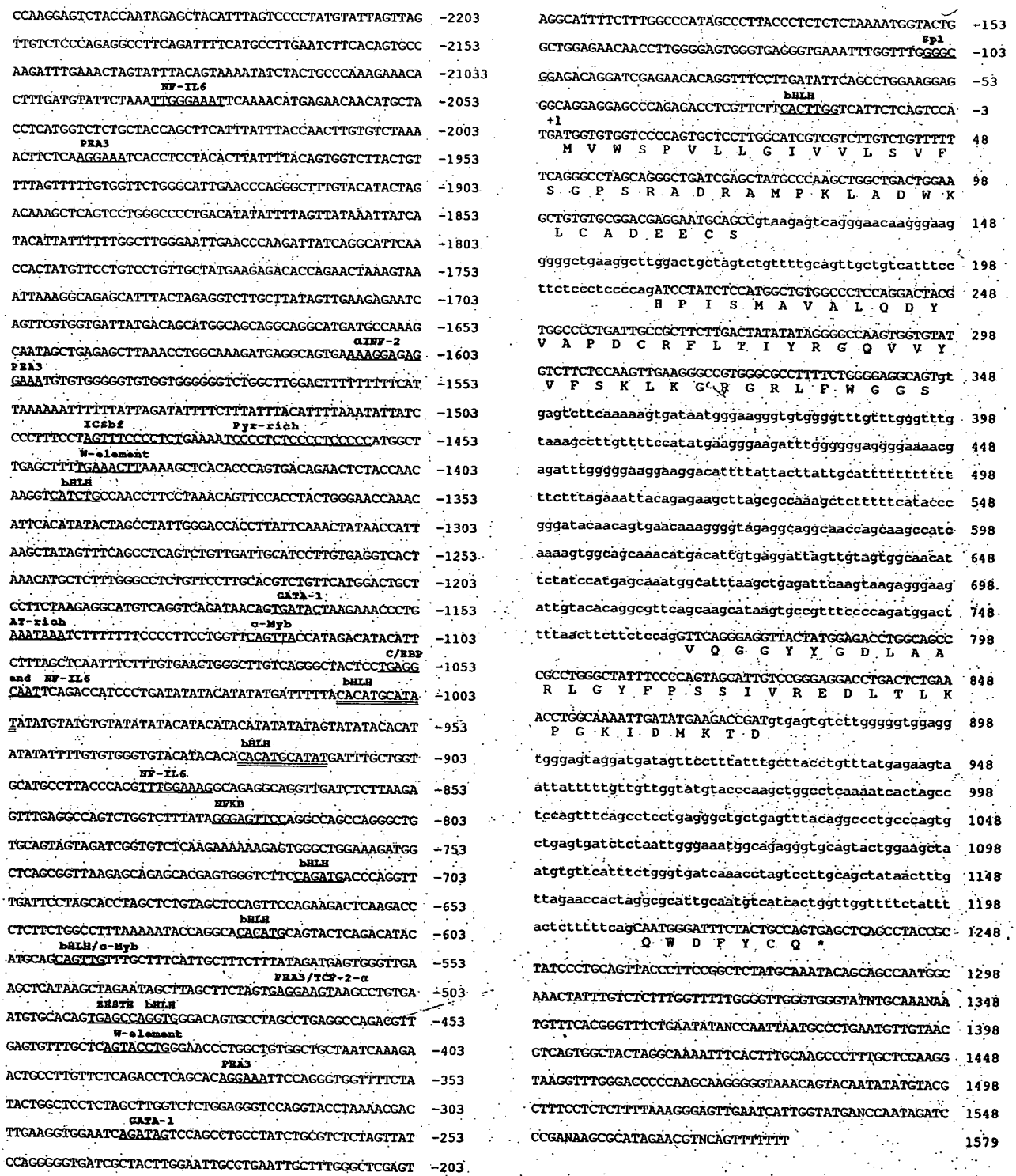

Fig. 4. Sequence of CD-RAP/MIA gene. Potential *cis*-acting sequences present in both the mouse and human genes are indicated by name and a single line. The bHLH sequences that are identical with the mouse type II procollagen gene are indicated by a double line. A star indicates the stop codon. Numbering begins at the start site of translation. (Genebank Accession #U85612)

chemistry was performed by the three-step avidin-biotin complex method developed by Hsu et al. (1981), using a DAKO LSAB 2-HRP Kit (Dako Corp. Carpinteria, CA) as directed by the manufacturer. In this procedure, the biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules, and the color reaction is developed using 3-amino-9-ethylcarbozole as a chromogen resulting in a red-colored precipitate at the antigen site. The antiserum was used at a dilution 1:400. Tissue sections were

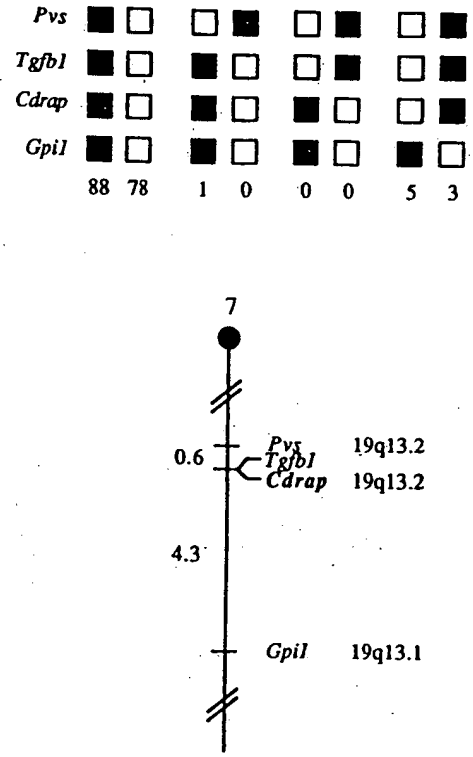

Fig. 5. *Cdrap* maps in the proximal region of mouse chromosome 7. *Cdrap* was placed on mouse chromosome 7 by interspecific backcross analysis. The segregation patterns of *Cdrap* and flanking genes in 175 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 175 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × *M. spretus*) F_1 parent. The shaded boxes represent the presence of a C57BL/6J allele and white boxes represent the presence of a *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 7 linkage map showing the location of *Cdrap* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

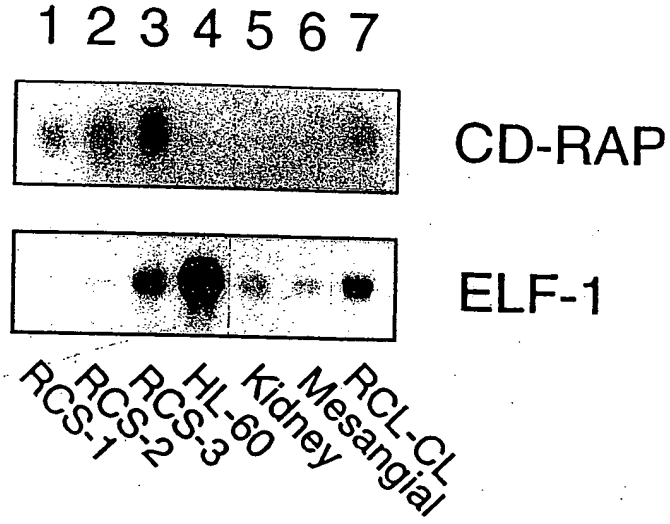

Fig. 6. Expression of CD-RAP/MIA in chondrosarcoma. **Upper panel:** Expression of CD-RAP/MIA (548 nts) in Swarm rat chondrosarcoma (**lanes 1–3**), leukemia cell line, HL-60 (**lane 4**), kidney (**lane 5**), mesangial cells (**lane 6**), and rat chondrosarcoma cell line (**lane 7**). **Lower panel:** The same northern blot was hybridized with a control probe, human elongation factor-1 (ELF-1) (1.2 kb).

observed and photographed with a Olympus BX40 microscope at 400× magnification.

Isolation of Genomic DNA

DNA was isolated from two mouse 129Sv strain libraries (Stratagene, La Jolla, CA), one in LambdaDashII, and the other in FIXII, using standard procedures (Sambrook et al., 1989). A mouse cDNA was used as a probe. From the FIXII library, the inserts of three independent phages were fully subcloned as *Sal*I fragments into pBluescript and 4 kb of the gene including 2,500 bases of the 5′ flanking region and all four exons were sequenced by Cycle Sequencing using the Dye Terminator Procedure (ABI, Foster City, CA). From the LambdaDashII library, one insert encompassing the entire gene plus 2,500 base pairs of 5′ flanking DNA was sequenced. Sequencing was performed on both strands of DNA. DNA from mouse mutants was obtained from Jackson Labs (Bar Harbor, ME) and included *pu* and Xs^J in the B6C3Fe strains. For genomic DNA, strands were sequenced by Cycle Sequencing (ABI) PCR amplification with Dye Terminator chemistry. DNA was analyzed using DNASIS (Hitachi Software).

Interspecific Mouse Backcross Mapping

Interspecific backcross progeny were generated by mating (C57BL/6J × *M. spretus*)F_1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N_2 mice were used to map the *cdrap* locus (see text for details). C57BL/6J and *M. spretus* mice were initially received from The Jackson Laboratory. The strains are maintained and the backcross was performed at the Frederick Cancer Research and Development Center, Frederick, MD. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (Jenkins et al., 1982). All blots were prepared with Hybond-N^+ nylon membrane (Amersham). The probe, an ~322-bp *Bam*HI/EcoRI fragment of rat cDNA (Dietz and Sandell, 1996) was labeled with [α^{32}P]dCTP using a random primed labeling kit (Stratagene); washing was done to a final stringency of 0.8 × SSCP, 0.1% SDS, 65°C. A fragment of 7.4 kb was detected in *Hinc*II-digested C57BL/6J DNA, and a major fragment of 19.0 kb was detected in *Hinc*II-digested *M. spretus* DNA. The presence or absence of the 19.0-kb *Hinc*II *M. spretus*-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Cdrap* including transforming growth factor,

beta 1 (*Tgf*β1) and glucose phosphate isomerase 1 complex (*Gpi*1) has been reported previously (Avraham et al., 1992). One locus, poliovirus sensitivity (*Pvs*), has not reported for this interspecific backcross. The probe, a 2.1-kb *Eco*RI fragment of mouse genomic DNA (kindly provided by Vincent Racaniello), detected major *Pvu*II fragments of 2.0 kb (C57BL/6J) and 1.4 kb (*M. Spretus*). The 1.4 kb *Pvu*II *M. Spretus*-specific fragment was followed in backcross mice. Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RNA Isolation

For isolation of total RNA from cell lines, the cells were harvested by centrifugation and dissolved in lysis buffer (4 M guanidinium/SCN, 25 mM Na-Citrate, 0.5% Na-Sarcosyl, and 0.7% β-mercaptoethanol). For preparation of total RNA from different soft tissues, fivefold volume lysis buffer was added to the tissue pieces, followed by homogenization of the tissues. Total cellular RNA was then isolated by CsCl centrifugation according to the method of Chirgwin et al. (1979).

Northern Blot Analysis

RNA was electrophoresed in an 1% agarose gel containing 6% formaldehyde and 1× MOPS buffer (1× MOPS: 20 mM morpholinopropane sulfonic acid, 5 mM Na-acetate, 1 mM EDTA). Transfer of RNA to Hybond-N nylon membranes was carried out by capillary blotting with 20× SSC (1× SSC: 0.15 M NaCl, 0.015 M Na-Citrate). After transfer, the nucleic acids were crosslinked to the membrane in a Stratalinker (Stratagene). RNA samples were kindly provided by Dr. Andrew Aprikian (HL60), Dawn Moran (mesangial cell), and Dr. Mike Harkey (rat chondrosarcoma cell line). Cells from the Swarm rat chondrosarcoma and the chondrosarcoma cell line were provided by Dr. James Kimura. For analysis of RNAs, cDNA probes were labeled with [^{32}P]-dCTP by specific priming and hybridized in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 100 mg/ml denatured salmon sperm DNA at 42°C for 16 hr (Ausubel et al., 1993). After hybridization, filters were washed twice in 2× SSC for 5 min at room temperature and twice in 2× SSC/0.1% SDS for 30 min each at 65°C. The washed filters were exposed to Hyperfilm-MP X-ray films. To determine the amount of mRNA bound to the Northern blot, the membrane was probed with cDNA encoding human elongation factor-1 as described previously (Hering et al., 1994).

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COVER PHOTOGRAPH: Immunolocalization of CD-RAP/MIA in clavicle cartilage of a 13.5 day mouse embryo. CD-RAP/MIA is a low molecular weight secreted molecule normally expressed exclusively by chondrocytes and pathologically expressed in melanoma and chondrosarcoma tumors. See Bosserhoff et al., *Developmental Dynamics* 208:516–525, 1997.

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Regulation of the Mouse Cartilage-derived Retinoic Acid-sensitive Protein Gene by the Transcription Factor AP-2*

(Received for publication, May 21, 1997, and in revised form, December 1, 1997)

Wei-Fen Xie‡, Seiji Kondo‡, and Linda J. Sandell‡§

From the Departments of Orthopaedics and Biochemistry, University of Washington and Veterans Administration Puget Sound Health Care Systems, Seattle, Washington 98108

The expression of cartilage-derived retinoic acid-sensitive protein (CD-RAP) is initiated at the beginning of chondrogenesis and continues throughout the cartilage development. In chondrocytes, CD-RAP is down-regulated by retinoic acid. To understand the molecular mechanism underlying this regulation and the cell-specific expression, the deletion constructs of the mouse CD-RAP promoter were transfected into chondrocytes and a melanoma cell line. The results revealed a domain that demonstrated high levels of expression specifically in chondrocytes. In this functional domain, we show that a *cis*-acting element, 5′-GCCTGAGGC-3′, binds to the *trans*-acting factor protein AP-2. Mutation of the AP-2 site on the CD-RAP promoter led to decreased transcription in C5.18 chondrocytes, indicating that this site may act as an activator of transcription. In contrast, increased concentration of AP-2, stimulated by retinoic acid, led to decreased transcription of the CD-RAP promoter, an effect that was abolished by mutation of the AP-2 binding site. The effect of AP-2 was further examined by co-transfection of C5.18 and HepG2 cells with the CD-RAP promoter constructs and an AP-2 expression plasmid. In a dose-dependent manner, cotransfection with AP-2 elevated and then decreased CD-RAP promoter activity. Taken together, these results suggest that AP-2 is involved in the biphasic regulation of CD-RAP transcription.

Cartilage-derived retinoic acid-sensitive protein (CD-RAP)[1] is a newly discovered secretory protein cloned from chondrocytes (1). Its human homologue, melanoma inhibitory activity (MIA), was originally isolated from the media of a highly metastatic melanoma and exerts autologous growth-inhibitory effects on melanoma cells *in vitro* (2, 3). CD-RAP is co-regulated with type II collagen by retinoic acid (RA) and is expressed exclusively by chondrocytes in adult rat and fetal bovine tissues (1). CD-RAP protein inhibits DNA synthesis in primary mature chondrocytes (4), but its normal function during chondrogenesis remains unknown.

CD-RAP was originally isolated as an mRNA down-regulated by RA in primary bovine articular chondrocyte cultures (1). Retinoids affect target genes by interacting with two families of ligand-dependent nuclear receptors, the RA receptors (RARα, -β, and -γ) and retinoid X receptors (RXRα, -β, and -γ) (5). Among the target genes identified for RA signaling are RARα3, RARβ isoforms, RARα2, and cellular retinoic acid-binding protein I and II genes. Synthesis of the transcription factor AP-2 is increased in response to RA with a lag period of 24–48 h in NT2 cells (6).

Eukaryotic gene expression is subject to the combined action of multiple DNA-binding proteins interacting with specific DNA motifs present in the promoters and enhancers. The transcription factor AP-2 recognizes the palindromic sequence 5′-GCCNNNGGC-3′ (7). AP-2 has been shown to play a crucial role in the control of gene expression in response to cell differentiation signals within neural crest and epidermal cell lineages (8). So far, three isoforms of the AP-2 gene have been identified from humans and mice that recognize the same binding motif (9, 10). Transcriptional regulation by AP-2 may involve both positive and negative regulatory effects on gene expression (11–13). Two independent groups have recently generated the mouse lines where the AP-2 gene has been disrupted by homologous recombination (14, 15). The AP-2 null mice died perinatally with cranio-abdominoschisis and severe dismorphogenesis of the face and skull, suggesting an AP-2 effect on the skeletal development.

Recent studies have shown that the human MIA/CD-RAP promoter directs a high level of gene expression specifically in human and murine melanoma cells (16). We have recently cloned and analyzed the mouse CD-RAP gene (17). In the present study, we evaluated the functional regulatory domains in the mouse CD-RAP promoter by transient transfection into chondrocyte and melanoma cell lines and demonstrated that an AP-2 binding motif may be a positive regulatory domain for chondrocyte-specific promoter activity. In contrast, the increase in AP-2 plays an important role in transcriptional reduction of CD-RAP in response to RA in chondrocytes. CD-RAP is the first gene specifically expressed in the skeletal development found to be regulated by AP-2. As CD-RAP is expressed during the chondrogenesis phase of endochondral bone formation, these findings may provide insight into the severe skeletal deformation observed in the AP-2-deficient animals.

EXPERIMENTAL PROCEDURES

Materials—The materials used in this work were purchased as follows: Dulbecco's modified Eagle's medium and Eagle's minimum essential medium from Bio-Whittaker; α-modified Eagle's minimum essential medium and restriction enzymes from Life Technologies, Inc.; fetal calf serum (FCS) from HyClone Laboratories, Inc.; pCMV-β-Gal from CLONTECH Laboratories, Inc.; luciferase expression vectors pGL3-Basic and pGL3-Control, β-galactosidase enzyme assay system, luciferase assay reagent, gel shift assay systems, and the reverse transcrip-

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‡ Present address: Washington University School of Medicine, Dept. of Orthopaedic Surgery and Dept. of Cell Biology and Physiology, 216 S. Kingshighway, Yalem Research Bldg., Room 704, St. Louis, MO 63110.

§ To whom correspondence should be addressed: Washington University School of Medicine, Dept. of Orthopaedic Surgery, Yalem Research Bldg., Room 704, 216 S. Kingshighway, St. Louis, MO 63110. Tel.: 314-454-7800; Fax: 314-454-5900; E-mail: sandelll@msnotes.wustl.edu.

[1] The abbreviations used are: CD-RAP, cartilage-derived retinoic acid-sensitive protein; MIA, melanoma inhibitory activity; RA, retinoic acid; FCS, fetal calf serum; RCS, rat chondrosarcoma; PCR, polymerase chain reaction; RT, reverse transcription.

TABLE I

Comparison of the mouse CD-RAP promoter activity in different cell types

The 2251-base pair mouse CD-RAP upstream flanking sequence was cloned into pGL3-B and transiently transfected into various cultured cells. The pGL3-Basic vector lacks eukaryotic promoter and enhancer sequences. The construct pGL3-C contains the SV40 promoter and enhancer sequences. The luciferase values are presented as mean ± S.E. C5.18, RCJ 3.1 C5.18 chondrogenic cell line; B16, melanoma cell line; 3T3, Balb/3T3 mouse fibroblasts.

Plasmids	Cell type			
	RCS	C5.18	B16	3T3
pGL3-B	0.18 ± 0.04	1.52 ± 0.89	32.46 ± 16.81	0.47 ± 0.27
pGL3-C	2317.00 ± 102.23	233.45 ± 96.61	2299.66 ± 414.58	508.50 ± 42.32
m2251	10.78 ± 3.56	72.04 ± 1.41	326.37 ± 45.11	0.61 ± 0.20
m2251/pGL3-B	59.9	47.4	10.0	1.3

tion system from Promega; [^{32}P]dCTP and [γ-^{32}P]ATP (3000 Ci/mmol) from NEN Life Science Products; Hybond-N membrane from Amersham Corp.; anti-AP-2 and anti-Sp1 antibodies from Santa Cruz Biotechnology; all-*trans*-RA from Sigma; poly(dI-dC)-(dI-dC) double-stranded DNA from Pharmacia Biotech Inc.; DOTAP liposomal transfection reagent from Boehringer Mannheim; and B16 (ATCC CRL 6322), HepG2 (ATCC HB-8065), and BALB/3T3 (ATCC CCL163) from ATCC. The rat calvaria chondrogenic cell line RCJ 3.1 C5.18 was kindly provided by Drs. Jane Aubin and Johan Heerche. The rat chondrosarcoma (RCS) cell line was provided by Dr. James H. Kimura. Human AP-2αA expression plasmid in pCMX-PL1 vector was provided by Dr. Reinhard Buettner. Human AP-2 expression plasmid SP-AP-2 and the control vector SP-NN were from Dr. Trevor Williams (7).

Cell Culture—RCJ 3.1 C5.18 cells were maintained in α-modified Eagle's minimum essential medium supplemented with 10% heat-inactivated FCS and 10 nM dexamethasone to preserve their cartilage phenotype (18). Primary chondrocytes were prepared from bovine articular cartilage as described by Kuettner *et al.* (19) and cultured in Dulbecco's modified Eagle's medium with 10% FCS as were B16 (mouse melanoma cell line), BALB/3T3 (mouse fibroblast), and RCS cells. HepG2 (human liver hepatoblastoma) cells were cultured in Eagle's minimum essential medium with 2.0 mM L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, Earle's buffered salt solution, and 10% FCS.

To investigate the RA effect on CD-RAP expression and protein binding, all-*trans*-RA was dissolved in absolute ethanol to obtain the 1000 × stock solution at various concentrations. An aliquot of the stock solution was added to the prewarmed medium, which was then added to the culture. The ethanol vehicle served as a control and was added to the medium to a final concentration of 0.1%.

Plasmid Constructs—To construct the CD-RAP promoter 5′-deletion constructs, the mouse CD-RAP template was amplified by polymerase chain reaction (PCR) with a 3′-antisense primer that bound at −3 relative to the CD-RAP translational start site in conjunction with different 5′-sense primers that bound at varying distances within the CD-RAP upstream flanking sequence. To facilitate subcloning of the amplified fragments, the antisense primer contained a *Hin*dIII restriction site adaptor, and the sense primer contained a *Sma*I site. The PCR fragments and the luciferase expression vector pGL3-Basic were digested separately with *Sma*I and *Hin*dIII before ligation. The nomenclature used for each deletion construct (shown in Fig. 1) indicates the number of base pairs of the upstream 5′-flanking sequence with respect to the ATG translation start codon. Site-directed mutagenesis within the CD-RAP promoter was performed by PCR (20). The wild type and the mutated DNA constructs were verified by DNA sequencing.

Transient Transfection and Luciferase Assay—DNA transfection of RCS cells was performed by electroporation as described by Mukhopadhyay *et al.* (21). C5.18, B16, HepG2, and 3T3 were transfected by the lipofection method. Briefly, the cells were cultured in the 35-mm dishes. Each cationic lipid/plasmid DNA suspension was prepared by mixing 2 μg of the luciferase reporter plasmid and 0.5 μg of the internal control plasmid pCMV-β-Gal with a solution of DOTAP in Hepes (20 mM, pH 7.4) according to the manufacturer's instructions. The cells were harvested 48 h later, and the lysate was analyzed for luciferase activity with a Turner TD 20e luminometer using Promega luciferase assay reagent. The galactosidase activity was measured with 50 μl of the lysate using the colormetric assay as described by the manufacturer. The luciferase activities were normalized to the β-galactosidase value. At least three independent transfection experiments were carried out for each construct. Data are presented as the mean ± S.E.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from the cultured cells by the method of Dignam *et al.* (22), except that all of the buffers were supplemented with protease inhibitors (1 μM phenylmethylsulfonyl fluoride, 1 mM each of leupeptin and pepstatin). For RA stimulation, the C5.18 cells were harvested after treatment with 1 μM of RA for 2 days.

Electrophoretic Mobility Shift Assay—Fragment A (between −401 and −548 relative to the mouse CD-RAP translational start site) was amplified by PCR. All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. The fragment A was end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Band shifts were performed by incubating 4 μg of the extract in the mobility shift buffer (2.5 μg of poly(dI-dC)-(dI-dC), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 50 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5) with the DNA probe. For the competition studies, the cold oligonucleotides were added at a 100-fold molar excess and incubated for 10 min at room temperature before adding the DNA probe. DNA protein complexes were resolved on a 6% nondenaturing polyacrylamide gel at 120 V for 2–3 h. For the antibody interference experiments, the antibody or preimmune serum was added to the reaction mixture and incubated for 30 min at room temperature before being analyzed on a polyacrylamide gel. The AP-2 antibody from Santa Cruz Biotechnology reacts with AP-2α but not with AP-2β or AP-2γ.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Northern Blot Analysis—Total RNA was isolated from C5.18 and B16 cells using Qiagen RNeasy Midi Kits. For the study of the RA effect on AP-2 expression, the C5.18 cells were treated with 1 μM of RA for 2 days before they were harvested. RT-PCR was performed as described previously (10). 2 μg of total RNA was used to synthesize the first strand. Primers 5′-CTGTTCAGTTCCGGGTCGCC-3′ and 5′-CGGTCCTGAGCCAGCAGG-3′ were added to amplify the AP-2αA and expected to yield a 426-base pair product. 10 μl of PCR products was electrophoresed on a 1.5% agarose gel. To facilitate quantification, DNA was transferred to Hybond-N membrane and hybridized with the ^{32}P-labeled specific human AP-2αA cDNA. Amplification of the β-actin mRNA served as a control as described by Covert and Splitter (23).

Northern blot was carried out as described previously (10). 8 μg of total RNA was loaded on 1.0% agarose formaldehyde gels and blotted onto nylon membranes. Hybridization was performed using radiolabled specific AP-2αA cDNA probe. The bands of the autoradiograph were quantitated by densitometry using the ISS SepraScan 2001™ 1-D system (ISS-Enprotech).

RESULTS

Mouse CD-RAP Is Expressed in Chondrocytes and Melanoma Cells—The 5′-flanking sequence of the mouse CD-RAP gene was fused to a promoterless luciferase reporter plasmid pGL3-Basic and tested for its ability to generate luciferase activity in the transiently transfected cells (Table I). As expected, the construct m2251 generated expression in melanoma cell line B16 and no expression in BALB/3T3. In addition, m2251 also demonstrated relatively high luciferase activity in chondrocytes and chondrosarcoma cells, suggesting that this CD-RAP upstream sequence contains the elements necessary for chondrocyte activity. Subsequent experiments were performed using RCJ 3.1C5.18 cells as the representative chondrocytes.

Deletion Analysis of the Mouse CD-RAP Promoter—The rat calvaria chondrogenic cell line RCJ 3.1 C5.18 cells maintain their cartilage phenotype under the appropriate conditions (18). The synthesis of type II collagen, aggrecan, and CD-RAP mRNA by C5.18 cells was confirmed by Northern blot analysis (data not shown). To identify the *cis*-acting elements mediating the mouse CD-RAP expression in chondrocytes, we transfected

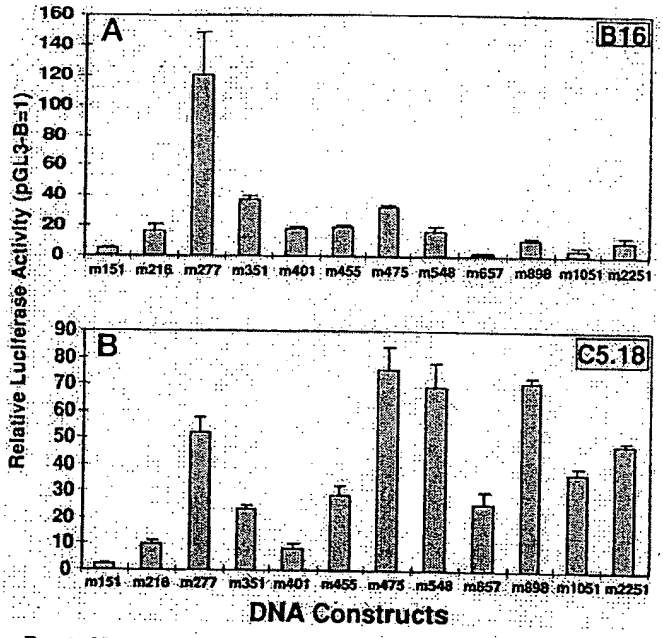

FIG. 1. **Nested deletion analysis of the mouse CD-RAP promoter.** The mouse CD-RAP promoter-luciferase reporter plasmids were cotransfected with pCMV-β-Gal control vector into B16 (*A*) and C5.18 cells (*B*). The luciferase activities were assayed 48 h later and normalized to the β-galactosidase value. The basal activity of the pGL3-Basic is set at 1.

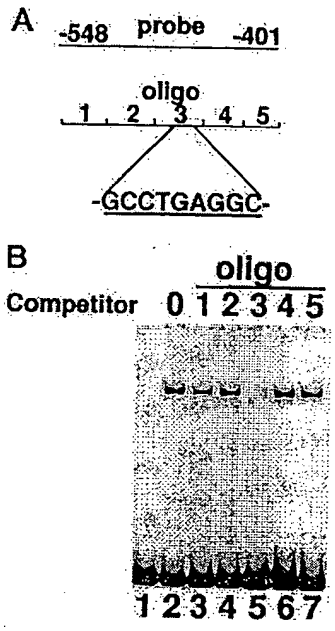

FIG. 2. **Competition experiments to identify the binding site of fragment A.** *A*, the relative location of various competitive oligonucleotides. The AP-2 motif in oligo 3 is *underlined*. *B*, competition was performed with different synthetic oligonucleotides using lysate from B16.

a series of the nested deletion CD-RAP promoter-luciferase expression plasmids into C5.18 and B16 cells and measured the luciferase activities generated in these cells. In B16 melanoma cells, the construct m277 generated the highest activity, while the activities of longer promoter constructs were consistently lower (Fig. 1*A*). In C5.18 cells, although the construct m277 could confer high activity, the longer constructs were also active, for example the construct m475 generated a 75-fold higher activity compared with the promoterless luciferase vector (Fig. 1*B*).

The luciferase activity was altered when upstream sequences of the CD-RAP promoter were deleted and transfected into the C5.18 cells, suggesting the presence of several positive and negative regulatory elements (Fig. 1*B*). Deletion of the promoter sequence from −1051 to −898 resulted in increased expression approximately 2-fold, identifying one or more silencer(s). The comparison of promoter activities generated by the constructs m657 and m548 revealed at least one negative regulatory element in this region. Deletion of the promoter sequence from −401 to −277 suggested another negative element. In contrast, truncation of the promoter sequences from −898 to −657, from −475 to −401, and from −277 to −216 showed a reduction of the promoter activity, suggesting the presence of at least three positive *cis*-acting sequences among these sites.

Identification of AP-2 Protein Binding Sites on CD-RAP Promoter—According to the transient transfection analysis, the addition of the sequences from −401 to −475 relative to the ATG protein start codon showed a remarkable increase in the promoter activities in C5.18 cells, implying that this region contained the positive regulatory elements for chondrocyte expression. In contrast, the luciferase activity of the constructs m401 and m475 varied slightly in the melanoma cells, and both of them expressed lower activities in B16 compared with the construct m277. We amplified a fragment (fragment A, −401 to −548) by PCR and performed gel mobility shift analyses to test whether this fragment binds to any protein factor that might regulate the CD-RAP promoter activity in chondrocytes. One strong DNA protein complex was observed with the B16 cell lysate (Fig. 2*B*, *lane 2*). An identical but fainter band was observed in the nuclear extracts of bovine articular cartilage and C5.18 cells (data not shown). To more precisely localize the DNA binding site, we carried out mobility shift assays using a series of synthetic oligonucleotides to compete against the labeled fragment A for protein binding. Fig. 2*A* shows the relative position of the five 30- or 20-mer oligonucleotide competitors. The competition was performed using the nuclear extract prepared from B16 cells. As shown in Fig. 2*B*, a 30-base pair competitor designated oligo 3 effectively inhibited the binding of the probe. The oligo 3 contains an AP-2 *cis*-acting motif as shown in Fig. 2*A*. The AP-2 site is located between −456 and −463 relative to the ATG protein start codon. To determine whether the protein binding could be competed by the known *cis*-acting sequences, we carried out gel shift analysis with the consensus oligonucleotides. The bandshift was inhibited by the consensus AP-2 oligonucleotide but not by the oligonucleotides containing AP1, Sp1, and OCT1 consensus sequences (data not shown), suggesting that AP-2 or a related protein bound to the CD-RAP promoter.

To determine which nucleotides of CD-RAP were important for the protein binding, mutations were introduced into the oligonucleotide competitors. Fig. 3*A* shows the sequences of oligo 3 with the wild type and the mutated AP-2 motifs. In the competition study, the mutations of GCC residues in the AP-2 core sequence (MuA and MuB) partially abolished the binding properties of the oligonucleotides, and they were not able to act as the effective competitors (Fig. 3*B*). In contrast, the oligo 3 still competed for the protein binding when mutated at T and A residues (MuC and MuD). This result is consistent with the AP-2 core recognition element for the AP-2 binding site, 5′-GCCNNNGGC-3′, where the internal 3 nucleotides are not critical for the binding (7). To confirm the binding of AP-2, a supershifted DNA-protein complex was obtained by incubating the labeled fragment A with the B16 extract and the antibody directed against AP-2. The preimmune serum did not alter the electrophoretic mobility pattern of the complex (Fig. 3*C*).

Functional Activity of AP-2 Sites—To investigate the func-

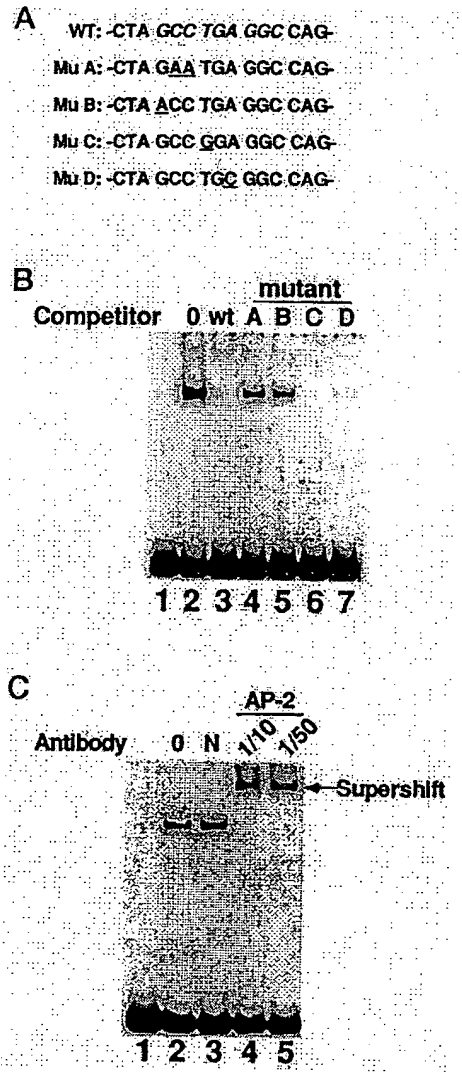

FIG. 3. **Identification of the AP-2 binding on CD-RAP promoter.** *A*, the sequences of the wild type and mutant oligo 3. The wild type (*WT*) oligo 3 contains the AP-2 binding motif (in *italics*). The mutations of AP-2 site are *underlined*. *B*, mutation analysis of the AP-2 binding site. Competitive electrophoretic mobility shift analysis of B16 extract was performed using fragment A as a labeled probe. The wild type (*lane 3*) and the mutant oligo 3 (*lanes 4–7*) were added to interfere with the protein binding. *Lane 1*, no nuclear extract; *lane 2*, no competitor. *C*, supershift assay with AP-2 antibodies. The preimmune serum (*lane 3*) or the antibodies against AP-2 were incubated together with B16 lysate and the labeled fragment A. Antibodies were used at two different dilutions: 1:10 (*lane 4*) and 1:50 (*lane 5*). *Lane 1* (no nuclear extract) and *lane 2* (containing nuclear extract) were incubated without serum. The complex was supershifted as indicated by the *arrow*.

tion of the *cis*-acting AP-2 motif in the CD-RAP promoter, we introduced the same mutations that abolished the binding activities in mobility shift experiments (MuA and MuB) by site-directed mutagenesis within the context of the strongly expressing reporter plasmid m475 and the longest promoter construct m2251. As shown in Fig. 4, the activities generated by the AP-2 mutant plasmid m2251-MuA decreased approximately 2-fold in comparison with its wild type promoter construct in C5.18 cells. In addition, the same mutation A within the construct m475 (m475-MuA) resulted in reduction of the promoter activity almost 3-fold. The construct m475-MuB also decreased the promoter activity. These results suggest that the AP-2 motif may act as a positive regulatory element necessary for the full expression of CD-RAP in chondrocytes under normal culture conditions. The remaining levels of CD-RAP promoter activity suggest that additional elements also contribute

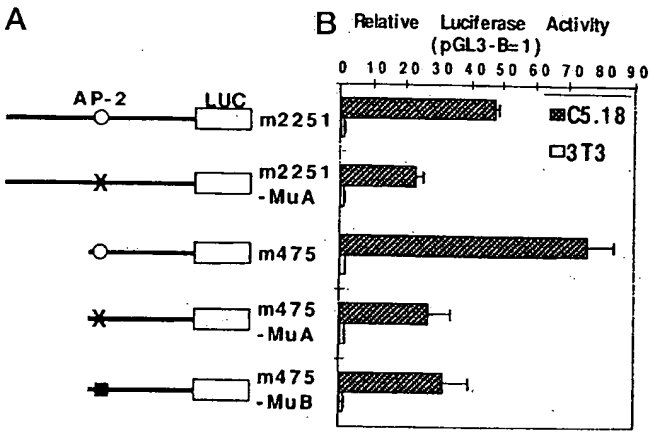

FIG. 4. **Mutation analysis of the AP-2 binding motif.** *A*, schematic diagram of the wild type and the mutated DNA constructs. The AP-2 binding site is indicated as an *open circle*. The mutated AP-2 motif is shown as a *cross* (mutation A) or *solid square* (mutation B), respectively. *B*, the luciferase activities conferred by the mutated plasmids in transiently transfected 3T3 (*open bar*) and C5.18 cells (*hatched bar*) were compared with the wild type constructs. The activity of promoterless plasmid pGL3-B was set at 1.

to its constitutive expression in chondrocytes.

Induction of AP-2 Expression by RA in C5.18—Since the previous experiment suggested that inhibition of the binding of AP-2 by mutagenesis of the AP-2 site reduced the CD-RAP transcription, and the binding of AP-2 in chondrocytes was faint in the gel shift assay, we expected that the constitutive concentration of AP-2 would be very low in C5.18. AP-2 gene expression is up-regulated in many cell types by the developmental morphogen, RA (6). We therefore speculated that RA might stimulate the AP-2 expression in chondrocytes, resulting in more AP-2 binding to the CD-RAP promoter. Semiquantitative RT-PCR was performed to investigate the effect of RA on AP-2 gene expression. Equal amounts of total RNA harvested from the C5.18 cells treated with ethanol control or RA were used. As shown in Fig. 5*A*, AP-2αA transcript levels were increased after 48 h of exposure to RA. Northern blot confirmed the stimulation of AP-2 expression in C5.18 cells by RA (Fig. 5*B*). Densitometric analysis revealed low levels of AP-2 RNA in the untreated C5.18 cells that increased 6-fold at 48 h of RA treatment. The AP-2 level in B16 cells was about 10 times higher than that in the untreated C5.18 cells.

We then conducted a gel shift assay using the C5.18 lysates derived from the cells treated with RA for 2 days. A DNA-protein complex appeared at the AP-2 corresponding position (Fig. 6). This complex was inhibited by the oligo 3, which contains the AP-2 binding site and a consensus AP-2 oligo, but not by the mutant A of the oligo 3 (CC → AA). A supershift complex was specifically induced by the antibody against AP-2. As a control, the Sp1 antibody was unable to shift the DNA-protein complex. These experiments confirmed that RA increased the AP-2 expression in C5.18 cells, leading to more binding to the CD-RAP promoter in the gel shift assay.

RA Reduces AP-2-dependent CD-RAP Gene Transcription—We have previously shown that RA down-regulates the CD-RAP expression (1). Since we now have shown that RA increases the AP-2 expression in C5.18 cells, we sought to determine whether AP-2 plays a role in the regulation of CD-RAP expression by RA. The wild type construct m2251, the mutated construct m2251-MuA, and the AP-2 deletion construct m455 were employed. RA or ethanol was added just prior to the transfection. RA caused a reduction of promoter activity in the construct m2251 in a concentration ranging from 10 nM to 1 μM (Fig. 7). Mutation of the AP-2 site (construct m2251-MuA) and deletion of the AP-2 site (construct m455) abolished

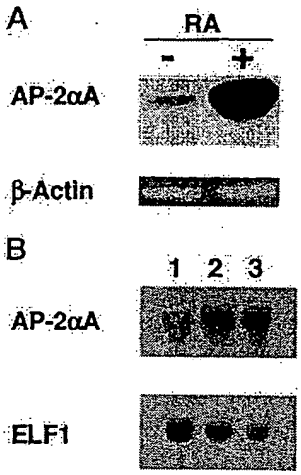

FIG. 5. **The RA effect on AP-2 in C5.18 cells.** *A*, total RNA was isolated from C5.18 cells incubated for 48 h with ethanol (−) or with 1 μM RA (+). RT-PCR was conducted using specific primers for AP-2αA. The PCR cycle is 28. The PCR products were separated on an agarose gel and transferred to nylon membranes. Hybridization was performed with AP-2αA cDNA probe. Amplification of β-actin served as a control for mRNA concentration (shown stained with ethidium bromide). *B*, total RNA isolated from C5.18 cells after 48 h of exposure to ethanol (*lane 1*) or 1 μM of RA (*lane 2*) and from B16 cells (*lane 3*) was analyzed by Northern blot hybridization with a radiolabled specific AP-2αA cDNA probe. As a control for RNA loading, hybridization was performed with a cDNA probe for ribosomal elongation factor 1 (ELF1).

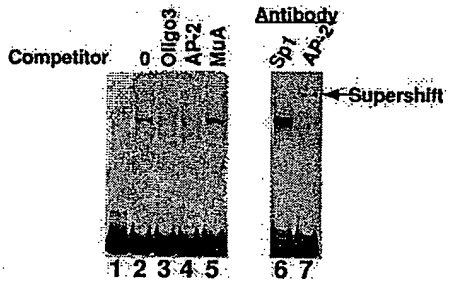

FIG. 6. **Induction of AP-2 binding by RA in C5.18 cells.** Mobility shift analysis was carried out with the extract from C5.18 treated with RA for 2 days. The specificity of AP-2 was confirmed by competition with oligo 3 spanning the AP-2 site on CD-RAP (*lane 3*), consensus oligo AP-2 (*lane 4*), and mutant A of oligo 3 (CC → AA, *lane 5*). Sp1 (*lane 6*) and AP-2 (*lane 7*) antibodies were added to supershift to the complex.

the ability of RA to inhibit CD-RAP gene expression. In addition, RA also inhibited the promoter activity of the construct m475 containing the AP-2 site but had no effect on the construct m415 lacking the AP-2 site (data not shown). This experiment strongly suggests that the AP-2 binding site is involved in the RA-induced reduction of the CD-RAP transcription.

Functional Effect of AP-2 on CD-RAP Promoter—We have shown that AP-2 may stimulate CD-RAP transcription, since mutation of the AP-2 motif reduces its promoter activity in C5.18 cells. On the other hand, high levels of AP-2 expression apparently inhibit the transcriptional activation of CD-RAP. The RT-PCR result revealed that the AP-2 transcript levels were significantly elevated upon RA treatment, and the increase in AP-2 with RA treatment is correlated with a decrease in CD-RAP promoter activity. These results suggest that AP-2 may have a biphasic effect on CD-RAP transcription dependent on the AP-2 concentration. To test this hypothesis, HepG2 cells that are considered to be AP-2-deficient (24) were transfected with the construct m2251 and a human AP-2 expression plasmid AP-2αA in pCMX-PL1 vector. In the absence of the AP-2 expression vector, the activity of m2251 was low in HepG2 cells. In contrast, CD-RAP activity was induced by AP-2 in a

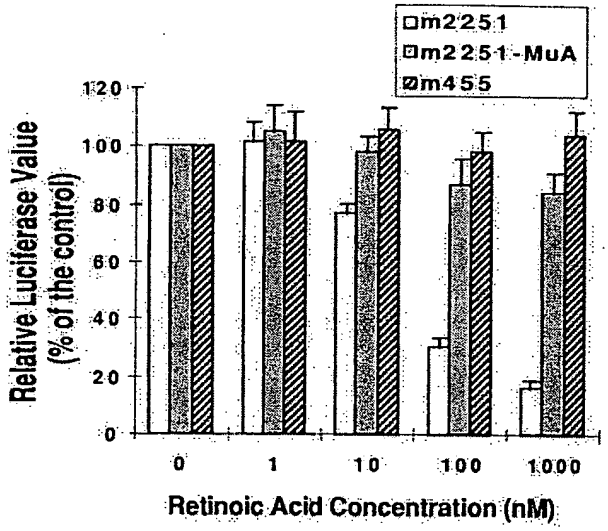

FIG. 7. **Inhibition of the CD-RAP expression by RA.** The CD-RAP promoter constructs were transfected into C5.18 cells. The constructs were wild type (m2251), mutated at AP-2 site (m2251-MuA), and deletion of AP-2 (m455). Different concentrations of RA or ethanol were added at the beginning of transfection. The luciferase activity was assayed 48 h later. The luciferase value without RA was taken as 100%.

dose-dependent fashion (Fig. 8*A*). As control, AP-2 had no effect on the promoterless construct pGL3-B and the construct m455, which does not include the AP-2-binding site (data not shown).

To determine whether expression of AP-2 affects the transcriptional activity of CD-RAP in C5.18 cells, the plasmid AP-2αA was cotransfected with the construct m2251. Fig. 8*B* shows that AP-2 slightly increased CD-RAP expression at low doses. Maximal activity was obtained at a dose of 0.25 μg of DNA/dish (35 mm). The CD-RAP expression was inhibited by high doses of AP-2. AP-2 reduced the CD-RAP promoter activity approximately 2-fold at a dose of 2.0 μg of AP-2αA DNA/dish. Similarly, another AP-2 expression plasmid (SP-AP2) also inhibited the CD-RAP promoter activities at high concentrations (data not shown). These results demonstrate that high expression of AP-2 results in the reduction of the CD-RAP promoter activity in C5.18 cells.

Last, to confirm that the exogenous AP-2 protein binds to the AP-2 DNA motif, we cotransfected the wild type and mutated CD-RAP promoter constructs into C5.18 cells along with 2 μg of human AP-2αA plasmid. 2 μg of AP-2 plasmid was shown above to decrease CD-RAP expression. As shown in Table II, the transcription of the wild type constructs m2251 and m475, both containing the AP-2 motif, was down-regulated more than 50% by AP-2. In contrast, mutation of the AP-2 binding site (m2251-MuA and m475-MuA) and deletion of AP-2 site (m455) abolished the AP-2 effect. This experiment confirms that the AP-2 motif is the primary site for AP-2 binding and is responsible for the reduction of the mouse CD-RAP transcription in C5.18 cells.

DISCUSSION

CD-RAP is thus far known to be expressed in chondrocytes (1, 17) and melanoma cells (2, 16). The function of CD-RAP is unclear, although it is thought to be involved in the regulation of DNA synthesis and cell shape (2). In this report, we present evidence for the involvement of the *trans*-acting factor, AP-2, in the regulation of CD-RAP transcription. These results show, for the first time, a functional role of AP-2 in cartilage differentiation and a potentially physiologically relevant RA effect mediated through AP-2 binding. The results are particularly significant in light of the recent reports of a severe skeletal phenotype resulting from disruption of the mouse AP-2 gene

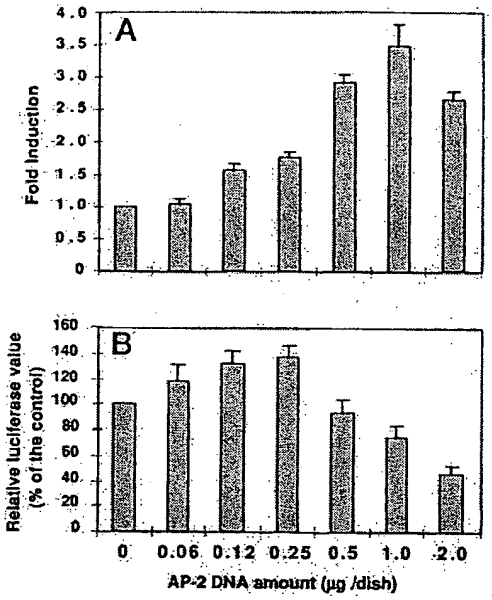

FIG. 8. **AP-2 effect on CD-RAP expression.** HepG2 (*A*) and C5.18 (*B*) cells were cotransfected with 2 μg of the construct m2251 and various amount of the human AP-2 expression plasmid AP-2αA in pCMX-PL1 vector. The differences in AP-2 DNA concentration were compensated for by control vector. The promoter activity was assayed 48 h later. The luciferase value of the control (without AP-2) was set as 1 in HepG2 cells and as 100% in C5.18 cells.

TABLE II
AP-2 effect on the CD-RAP expression: effects of the mutated AP-2 cis-acting sequences

A concentration of AP-2 shown to be inhibitory (Fig. 8) was used. The wild type constructs (m2251 and m475), the mutated constructs (m2251-MuA and m475-MuA), and the AP-2 deletion constructs (m455) were cotransfected into C5.18 cells with 2 μg of the human AP-2αA plasmid or a control plasmid, pCMX-PL1.

Constructs	AP-2-induced reduction
	%
m2251	54 ± 6.7
m475	58 ± 8.1
m2251-MuA	11 ± 3.8
m475-MuA	5 ± 2.3
m455	0 ± 0.1

(14, 15).

AP-2 functions in mediating the regulation of gene expression in response to a number of different signal transduction pathways. Phorbol esters and cyclic AMP induce AP-2 activity independent of protein synthesis (25). RA induces AP-2 activity by increasing AP-2 mRNA levels in human teratocarcinoma cells (6) and P19 embryonic carcinoma cells (9). In addition, AP-2 is associated with the programmed gene expression during retinoid-controlled murine embryogenesis (8). Restricted spatial and temporal expression patterns of AP-2 have been detected in several embryonic tissues, in particular in neural crest-derived cell lineages and in limb bud mesenchyme during the developmental stage when they are known to be retinoid-sensitive. Analyses of AP-2 expression in the embryonic and adult *Xenopus* tissues suggest a role for AP-2 in regulation of keratin gene expression during skin differentiation (26). Our deletion analysis of the CD-RAP promoter identified a fragment that includes an AP-2 motif and generates a high level of expression specifically in chondrocytes. Mutation or deletion of this AP-2 site led to decreased CD-RAP transcription in the transiently transfected chondrocytes, indicating that AP-2 may act as an activator of transcription for CD-RAP. Since mutation of the AP-2 site did not completely inhibit CD-RAP transcription, factors other than AP-2 may also be important in chondrocyte expression. Sox9 has recently been shown to be involved in the control of the cell-specific activation of *COL2A1* in chondrocytes and to directly regulate the type II collagen gene *in vivo* (27, 28). Gel shift and mutation analysis have identified a potential protein binding site for Sox9 at −409, and an Sp1 binding site at −100. The role of these sites in CD-RAP transcription is currently under investigation.

RA plays an important role in the regulation of growth during embryonic development and cell differentiation. RA is involved in induction and morphogenesis of limbs (29) and, in excess, is a potent teratogen predominantly deforming limbs, craniofacial structures, and the central nervous system (30–32). RA also participates in the regulation of chondrocyte metabolism during endochondral ossification of the growth plate. Pacifici and colleagues (33) have shown that 10–100 nM RA stimulates the maturation of chondrocytes including causing growth plate chondrocytes in culture to flatten, thus favoring cell adhesion and spreading (33). RA clearly suppresses CD-RAP expression in chondrocytes at the mRNA level (1), and we show here that it inhibited the CD-RAP gene transcription. Mutation or deletion of the AP-2 site abolished the RA effect on CD-RAP expression, suggesting that AP-2 plays a critical role in the RA-induced down-regulation of CD-RAP in chondrocytes.

AP-2 can both activate and inhibit gene expression in genes other than CD-RAP. For example, a biphasic response has been observed in the insulin-like growth factor-binding protein-5 gene after cotransfection of AP-2 expression plasmid with the insulin-like growth factor-binding protein-5 promoter construct. Our results showed that exogenously added AP-2 expression vector increased transcription of the transfected CD-RAP promoter in AP-2-deficient HepG2 cells. In chondrocytes, due to endogenous AP-2 expression, the addition of low levels of AP-2 exerted only slight activation of the CD-RAP promoter, while higher amounts of AP-2 inhibited CD-RAP promoter activity. Altogether, these results suggest that AP-2 may play a dual role in the control of CD-RAP expression. At low levels, as occur constitutively in chondrocytes, it may activate CD-RAP expression; at high levels, as occur with RA treatment, it suppressed CD-RAP expression.

AP-2 has been shown to inhibit gene transcription by three mechanisms. First, an alternative splice product of AP-2αA, called AP-2αB, contains the activation domain of AP-2 and part of the DNA binding domain but lacks the dimerization domain necessary for DNA binding. AP-2αB is a potent inhibitor of transactivation by AP-2αA by interfering with binding of AP-2αA with DNA (34). Second, AP-2 can inhibit transactivation of Myc by competing with an overlapping binding site as well as binding directly to Myc and impairing DNA binding of the Myc-Max complex (11). Third, high levels of AP-2 can down-regulate gene expression by the mechanism of "self-interference" (12). Self-interference is thought to occur when excess AP-2 molecules interact with one or more putative AP-2 cofactors, making them unavailable for AP-2 function (12). Since there is no AP-2αB as detected by RT-PCR in the chondrocytes (data not shown) and no obvious overlapping *cis*-acting sequence and the RT-PCR and Northern blot analysis revealed that the AP-2 expression was significantly increased after RA treatment, we speculate that down-regulation of the CD-RAP promoter activity in chondrocytes by RA is due to the "self-interference" of AP-2.

AP-2 recently, and unexpectedly, has been associated with the lethal skeletal defects in mice (14, 15). The AP-2 knockout mice exhibited anencephaly, craniofacial defects, and thoracoabdominoschisis. In the trunk and head, many bones were deformed or absent. The expression of skeletal patterning

genes *Pax-3*, *twist*, and *Msx-1* (14) was normal, indicating that they were unaffected by removal of AP-2. Taken together with the involvement of AP-2 in the regulation of the CD-RAP gene expression, it is possible that this cartilage gene is abnormally expressed in the AP-2-deficient animals. We have generated a transgenic mouse line harboring the 2.2-kb mouse CD-RAP promoter used in the present studies, which shows chondrocyte-specific expression.[2] Mutations of the AP-2 motif in the CD-RAP promoter will be made using transgenic mice to clarify the role of AP-2 in CD-RAP expression *in vivo*.

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The 2.2-kb promoter of cartilage-derived retinoic acid-sensitive protein controls gene expression in cartilage and embryonic mammary buds of transgenic mice

Wei-Fen Xie, Xin Zhang, Linda J. Sandell*

Washington University School of Medicine, Department of Orthopaedic Surgery, Yalem Research Building, Room 704, 216 South Kingshighway, St. Louis, MO 63110, USA

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Abstract

Cartilage-derived retinoic acid-sensitive protein (CD-RAP) is a secreted protein primarily expressed in chondrocytes. Pathologically, CD-RAP is detected in melanoma, chondrosarcoma and breast cancer. As an approach to define the transcriptional regulatory domains responsible for induction of chondrocyte activity in vivo, we generated transgenic mice harboring various fragments of the mouse CD-RAP promoter linked to the *Escherichia coli* β-galactosidase gene. Analysis of the transgene expression pattern by X-gal staining indicates that 2251 bp of the CD-RAP 5′-flanking sequence generates β-galactosidase activity in all cartilage in embryos and adult animals. In addition, we also detected transient X-gal staining in mammary gland primordium from day 11.5 to 15.5 of gestation. Histological examination revealed that the transgene is located in the chondrocytes of cartilage and the epithelial cells of mammary buds. The cartilage transgene expression pattern is consistent with that of endogenous CD-RAP gene expression. The presence of β-galactosidase in the mammary buds led us to the demonstration of a unique pattern of transient endogenous expression of CD-RAP in the mammary bud. The finding of transient CD-RAP expression in mammary buds suggests that it may play a role in the organogenesis of mammary glands.

Keywords: Cartilage-derived retinoic acid-sensitive protein; Melanoma inhibitory activity; Cartilage; Mammary buds; Transgenic mice

1. Introduction

Cartilage-derived retinoic acid-sensitive protein (CD-RAP) was identified in bovine chondrocytes by reverse transcription-polymerase chain reaction (PCR) and differential display during a screen to identify the molecular markers in the regulation of chondrogenesis (Dietz and Sandell, 1996). The protein was previously isolated and cloned as melanoma inhibitory activity (MIA) from a human melanoma cell line (Blesch et al., 1994). CD-RAP MIA is a small secreted protein activated from the beginning of chondrogenesis and expressed throughout cartilage development (Dietz and Sandell, 1996; Bosserhoff et al., 1997a). Unlike other cartilage genes such as collagen types II, IX and XI, which are also observed in a variety of non-cartilaginous tissues during embryogenesis (Cheah et al., 1991; Sandell et al., 1991, 1994; Yoshioka et al., 1995), the normal expression of CD-RAP MIA is more restricted.

Evidence suggests that CD-RAP MIA plays an important role in carcinogenesis and tumor metastasis as CD-RAP MIA may mediate the detachment of

* Corresponding author. Tel.: -1-314-454-7800; fax: 1-314-454-5900.

E-mail address: sandell1@msnotes.wustl.edu (L.J. Sandell).

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melanoma cells from extracellular matrix molecules (Bosserhoff et al., 1997b,c). CD-RAP MIA is detected at various levels in malignant melanoma, chondrosarcoma and mammary carcinomas (Blesch et al., 1994; van Groningen et al., 1995; Bosserhoff et al., 1997a,b; Lu et al., 1997). Treatment of a melanoma cell line HTZ-19 with MIA resulted in inhibition of DNA synthesis and significant change of cell morphology as melanoma cells rounded up (Blesch et al., 1994). Increased serum levels of CD-RAP MIA were detected in 100% of the patients with metastatic melanoma. In contrast, none of the patients with negative CD-RAP MIA values developed metastasis, suggesting that CD-RAP MIA may participate in tumor invasion and metastasis (Bosserhoff et al., 1997b). In addition, CD-RAP MIA is a novel serum marker for malignant melanoma with higher sensitivity and specificity than S-100 protein and soluble intercellular adhesion molecule 1, the common markers for melanoma. The CD-RAP MIA serum level is being used for the staging and monitoring of metastatic melanoma (Bosserhoff et al., 1997b).

The type II collagen gene has been used by several groups to investigate the transcriptional mechanism of chondrogenesis (Horton et al., 1987; Ryan et al., 1990; Savagner et al., 1990; Bell et al., 1997; Lefebvre et al., 1997, 1998a; Zhou et al., 1998). Because of its restricted expression pattern, CD-RAP, like type II collagen, is also a good model for studies on the transcriptional regulation of cartilage specific genes. The gene encoding CD-RAP MIA is well conserved among different species and is comprised of four exons interrupted by three introns (Dietz and Sandell, 1996; Bosserhoff et al., 1997a). Functional analysis of the CD-RAP MIA promoter shows that the gene is actively expressed in human and murine melanoma cells and its activity is inducible by phorbol ester (Bosserhoff et al., 1996). Our previous studies demonstrated that 2251 bp of the mouse CD-RAP 5′-flanking sequence contains the *cis*-acting elements for the activation of chondrocyte-specific gene expression in vitro (Xie et al., 1998). This promoter contains a TATA box and the binding sites for transcription factors such as Sp1, LEF-1, AP-2, Sox9, and bHLH proteins. In particular, AP-2 and Sox9 have been shown to be important for chondrocyte differentiation (Foster et al., 1994; Wright et al., 1995; Schorle et al., 1996; Zhang et al., 1996; Ng et al., 1997; Zhao et al., 1997; Lefebvre et al., 1998a). We have shown before that AP-2 is biphasically involved in the regulation of CD-RAP promoter activity and its response to retinoic acid, i.e. the *trans*-activation by AP-2 contributes to the constitutively high expression of CD-RAP in chondrocytes and overexpression of AP-2 induced by retinoic acid results in significant reduction of the CD-RAP transcript (Xie et al., 1998). In addition, Sox9 binds to the CD-RAP promoter and enhances CD-RAP expression (Xie et al., 1999). However, it appears that additional transcription factors are essential for the activation of chondrocyte differentiation, since neither Sox9 nor AP-2 is able to stimulate endogenous expression of cartilage genes in non-chondrogenic cells (Lefebvre and de Crombrugghe, 1998b; Xie et al., 1999).

In this study, we attempt to define the DNA domain containing the *cis*-acting elements that can target correct spatiotemporal expression of CD-RAP in vivo during embryonic development. Transgenic mice harboring various lengths of the mouse CD-RAP promoter linked to the *E. coli* β-galactosidase gene (*lacZ*) were generated. The results indicate that the mouse CD-RAP promoter from 2251 to 3 contains the transcriptional regulatory elements that are sufficient for the tissue-specific expression in vivo. In addition to cartilage expression, CD-RAP is transiently expressed in mammary buds of embryos, thereby setting the stage for subsequent reexpression during breast carcinogenesis.

2. Results

2.1. Production of transgenic mice

Our recent transient transfection analysis revealed that 2251 bp of the mouse CD-RAP promoter contains chondrocyte-specific regulatory domains (Xie et al., 1998). To determine whether the mouse CD-RAP promoter can target correct spatio-temporal expression in vivo and to define the transcriptional regulatory elements that are responsible for the chondrocyte-specific expression, three different lengths of the mouse CD-RAP promoter were fused to the *lacZ* reporter gene (Fig. 1).

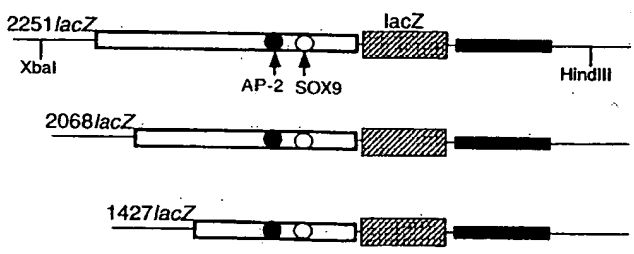

Fig. 1. Diagram of the constructs used for generation of transgenic mice. Three different lengths of the mouse CD-RAP upstream promoter DNA were linked to the *Escherichia coli* ß-galactosidase gene (lacZ) followed by an intron and a polyadenylation signal (polyA) of the murine protamine gene (solid box). Empty boxes represent the mouse CD-RAP promoter DNA and hatched boxes donate the lacZ DNA. The restriction sites used for releasing the constructs and the binding sites for transcription factors AP-2 (•) and Sox9 ( ) are shown.

The fusion genes were microinjected into the fertilized eggs to generate transgenic mice. Two of the five transgenic lines harboring the 2251 *lacZ* (Nos. 152 and 156) exhibited detectable β-galactosidase activity with X-gal staining. The founder transgenic mice were mated with the B6SJL wild type mice to establish transgenic lines, allowing analysis of the transgene expression at different embryonic stages. No variation in the pattern and the intensity of the transgene expression was observed in different embryos of the same established line and between these two positive transgenic lines.

A total of 21 transgenic founder mice carrying 2068 *lacZ* or 1427 *lacZ* were obtained. None of the offspring of these founders revealed any detectable β-galactosidase activity during the different embryonic stages. Subsequent experiments were carried out using the established transgenic line No. 152 carrying 2251 *lacZ* and demonstrating positive X-gal staining.

2.2. Detection of transgene expression during murine development

To investigate the spatio-temporal expression pattern of the transgene, transgenic embryos were collected at days 10.5, 11.5, 12.5, 13.5, 15.5 and 16.5 of gestation by cesarean section and whole mount-stained with X-gal. The earliest positive staining was observed in the forelimb bud at day 10.5 post-conception (p.c.) (data not shown). At day 11.5 p.c., 2251 *lacZ* began to generate expression in cartilage of the developing bones including mandible and scapula consistent with previous results by in situ hybridization (Bosserhoff et al., 1997a). The proximal part of forelimb was also slightly stained at this stage which is correlated with the advent of the differentiation of limb buds (Fig. 2A). We also detected faint staining in mammary buds at this stage. In both males and females, the transgene expression in mammary gland primordium continued throughout day 15.5 p.c. with the strongest expression observed at day 13.5 p.c. (Fig. 2A–D).

At day 12.5 p.c., intense X-gal staining was observed in the primordial cartilage of large diarthroses, including shoulder, elbow, knee, and ankle articulations, while faint staining was also seen in the cartilage anlages of long bones and phalanges in the limbs (Fig. 2B). The transgene expression in cartilage became

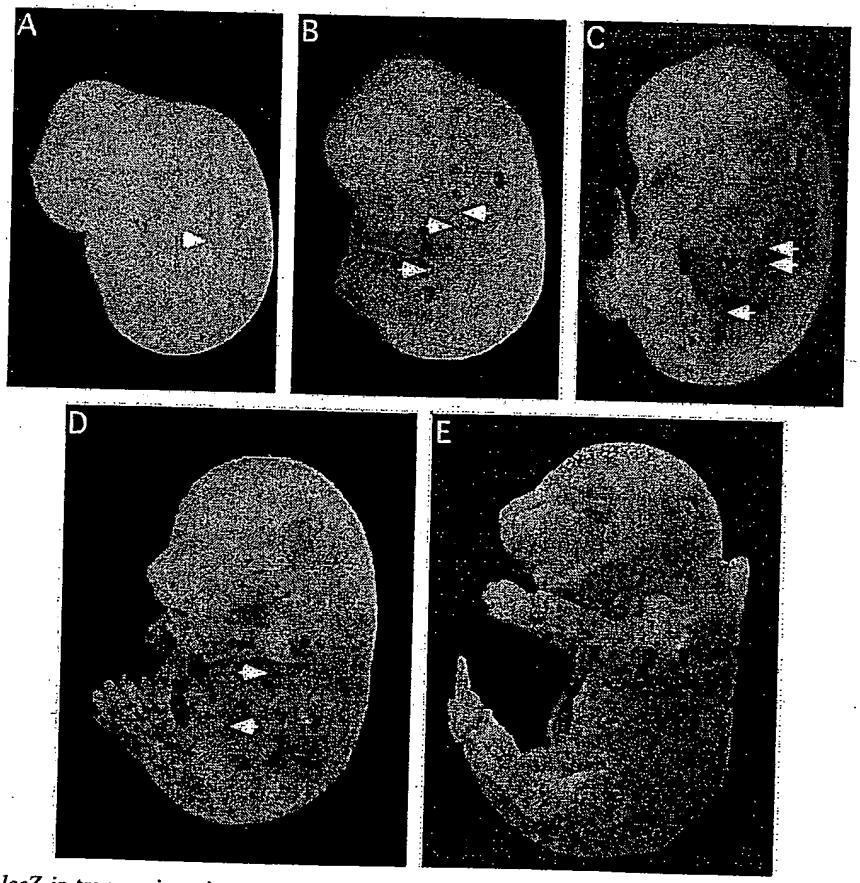

Fig. 2. Expression of 2251*lacZ* in transgenic embryos at different stages of development. The embryos were collected by cesarean section at days 11.5 (A), 12.5 (B), 13.5 (C), 15.5 (D), and 16.5 (E) of gestation and whole mount-stained with X-gal. The β-galactosidase activity was observed in the mammary gland primordia (arrows) and cartilage.

more evident at day 13.5 p.c. (Fig. 2C). The vertebral column, scapula, clavicle and nasal cartilage and several craniofacial structures were clearly stained with X-gal at day 13.5 p.c. As chondrogenesis proceeded to the proximal phalanges by day 15.5 p.c., the *lacZ* expression was clearly detected in the distal cartilage and the proximal articulations, while the expression in mammary gland primordium was decreased (Fig. 2D). We also detected X-gal staining on the tip of genital tubercle at this stage (data not shown). No staining in mammary gland primordium of either males or females was observed at day 16.5 p.c., while the transgene expression in cartilage remained almost the same intensity as that of day 15.5 embryo (Fig. 2E). No X-gal staining was detected in skin, somites or notochord at anytime during the embryonic development.

2.3. Histological analysis

Whole mount-staining demonstrated that the 2251 bp of the mouse CD-RAP promoter contains the *cis*-acting elements, which are sufficient for generating *lacZ* expression in cartilaginous tissues and mammary gland primordium. To determine the nature of cells that contained the β-galactosidase activity, the stained transgenic embryos were sectioned and the tissue sections were counterstained with eosin. Histological examination confirmed that the transgene was expressed in essentially all cartilage tissues (Fig. 3). Positive X-gal staining was observed in chondrocytes of ribs, vertebral body, limb buds, otic vesicle, Meckel's cartilage, nasal cartilage, femur and hyoid body. No *lacZ* expression was detected in heart, liver or kidney tissues. In ribs (Fig. 4A) and forelimb (Fig. 4B) of day 15.5 p.c., 2251 *lacZ* targeted strong expression primarily in proliferating chondrocytes, while the expression was reduced in hypertrophic chondrocytes. In adult animals, the transgene still generated *lacZ* expression in cartilage (Fig. 5). Clear X-gal staining was detected in the articular cartilage and growth plate of an 8-week knee joint section (Fig. 5A,B). No detectable *lacZ* expression was identified in non-cartilaginous tissues known to synthesize type II procollagen mRNA such as somites, mesenchymal and epithelial cells, notochord, heart, kidney, and discrete areas of the brain (Cheah et al., 1991; Sandell et al., 1991, 1994),

To compare the expression pattern of transgene and endogenous CD-RAP gene, specific antibody was employed to localize CD-RAP. The results strongly suggested that the pattern of transgene expression is the same as that of endogenous CD-RAP gene in both embryos and adult animals. Fig. 5 shows both CD-RAP (Fig. 5A,B) and transgene (Fig. 5C,D) expression were detected in articular cartilage of an 8-week joint section. Similarly, CD-RAP was observed in cartilaginous lung bronchi and rib chondrocytes of 13.5-day embryo (Fig. 6B) in which *lacZ* transgene expression was also detected (Fig. 6A).

Microscopic observation also revealed that all the epithelial cells in mammary buds were intensely stained from day 11.5 to 15.5 p.c. (Fig. 7A,B) as suggested in the whole mount embryo staining. To demonstrate endogenous CD-RAP expression in mammary buds, we performed immunohistochemistry to localize CD-RAP using a 13.5-day-old embryo (Fig. 7C). CD-RAP was detected in the cytoplasm of mam-

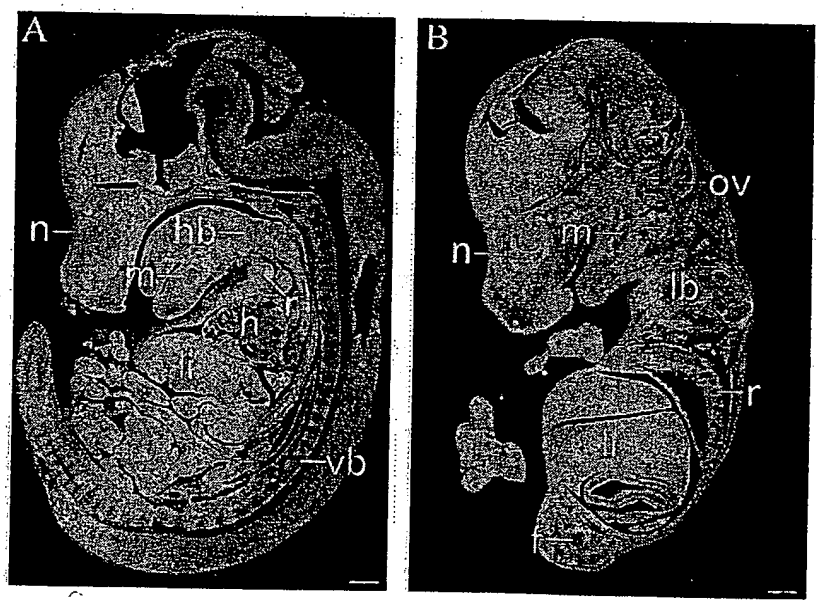

Fig. 3. Darkfield image of a 13.5-day embryo stained for β-galactosidase expression. Strong transgene expression (pink) was detected in cartilage of a medial section (A) and a sagittal section (B). Abbreviations: f, femur; h, heart; hb, hyoid bone; lb, limb buds; li, liver; m, Meckel's cartilage; n, nasal cartilage; ov, otic vesicle; vb, vertebral body; and r, ribs. Bars, 200 μm.

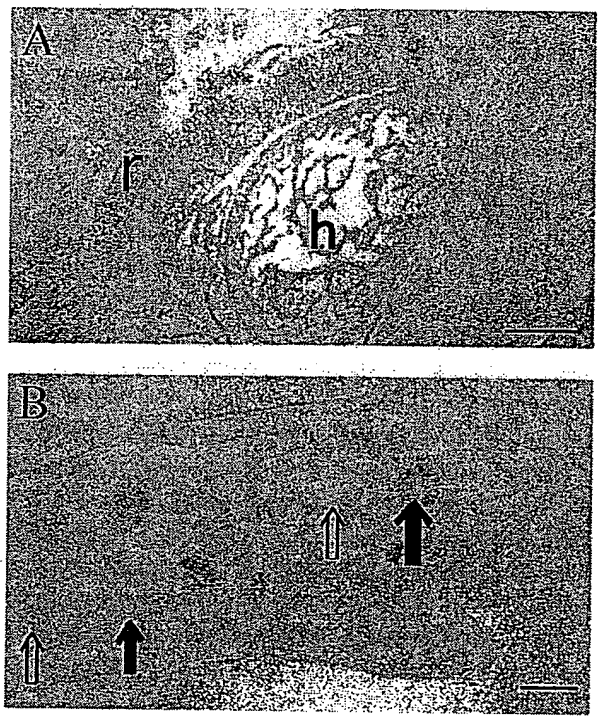

Fig. 4. Brightfield image of histological analysis of transgene 2251*lacZ* expression. (A) Expression of the *lacZ* constructs in cartilage. X-gal staining was positive in the chondrocytes of rib (r) from a 13.5-day p.c. embryo. No expression was detected in heart (h). (B) Section of the forelimb of day 15.5 p.c. The β-galactosidase activity was located in proliferating chondrocytes (filled arrow). Transgene expression in hypertrophic chondrocytes was decreased (empty arrow). Bars, 100 μm (A); 200 μm (B).

mary epithelial cells, suggesting that the transgene expression in mammary gland primordium is consistent with that of the endogenous CD-RAP gene.

3. Discussion

The current study demonstrates that the 2251 bp of mouse CD-RAP promoter used in the *lacZ* constructs confers both temporal and spatial tissue-specific expression. Our previous studies have shown that CD-RAP has a more restricted expression pattern than other cartilage genes such as collagen types II, IX, and XI (Cheah et al., 1991; Sandell et al., 1994; Yoshioka et al., 1995; Bosserhoff et al., 1997a). The 2251 *lacZ* transgene generates β-galactosidase activities in all cartilage tissues. The expression of CD-RAP transgene in chondrocytes begins at 10.5 days p.c. and continues through the adult animals. No expression was detected in other non-cartilaginous tissues such as skin, somites, notochord, heart, liver and kidney where type II collagen is transiently expressed. Histological examination and immunostaining confirms the expression specificity and fidelity. Both transgene and CD-RAP were observed in chondrocytes and mammary epithelial cells. No detectable β-galactosidase activity was identified in osteogenic and bone cells, nor in neuroepithelium, periosteum, and perichondrium.

CD-RAP is detected in human breast cancer (Bosserhoff et al., 1998) and Lu and colleagues isolated CD-RAP MIA as one of the few molecules that were differentially expressed in chemically induced rat mammary tumors (Lu et al., 1997). No expression has been detected in normal breast tissue, pregnant tissue or lactating mammary gland (Lu et al., 1997), therefore, our finding of embryonic expression provides the first example of CD-RAP expression in normal mammary tissue. In normal mouse development, the mammary ridge begins to appear on both sides of the ventral midline on day 11 of gestation. Five buds subsequently appear on each side (one in humans), three thoracic and two inguinal. The mammary buds are formed by epithelial ectoderm cells that collect and protrude into the mesenchyme. Both males and females develop in the same way through the mammary bud stage. In the female, in the days prior to birth, the epithelial cells of the bud begin to proliferate rapidly giving rise to the mammary cord which opens at the skin to form the nipple at one end and branches into ducts at the other end (Gilbert, 1994; Sakakura, 1987). After day 15 p.c. in the male, mesenchyme condenses around the center the mammary bud and the cells die. In culture with testosterone, mammary buds degenerate through signals generated from the mesenchyme (Kratochwil and Schwartz, 1976). As CD-RAP expression is first observed on day 11.5 and ceases after day 16.5, it coincides with the development of the mammary bud and not with subsequent proliferation and formation of the mammary cord. Because of this pattern of expression in the embryo and tumor, we speculate that CD-RAP MIA may act as a feto-oncogene. This is the first report of CD-RAP expression in premammary gland and the only DNA promoter sequence known to be capable of inducing this pattern of expression.

The understanding of skeletal development has been considerably advanced in the past decade. Several transcription factors that activate the differentiation of myoblasts, adipocytes, and osteoblasts have been identified (Davis et al., 1987; Tontonoz et al., 1994; Molkentin et al., 1995; Kim and Spiegelman, 1996; Ducy et al., 1997). However, our current knowledge of the chondrocyte differentiation process is still limited. AP-2 and Sox9 have been shown to be involved in skeletal development. The importance of AP-2 in chondrogenesis is demonstrated in the AP-2 knock out mice which reveal severe skeletal deformation (Schorle et al., 1996; Zhang et al., 1996). CD-RAP

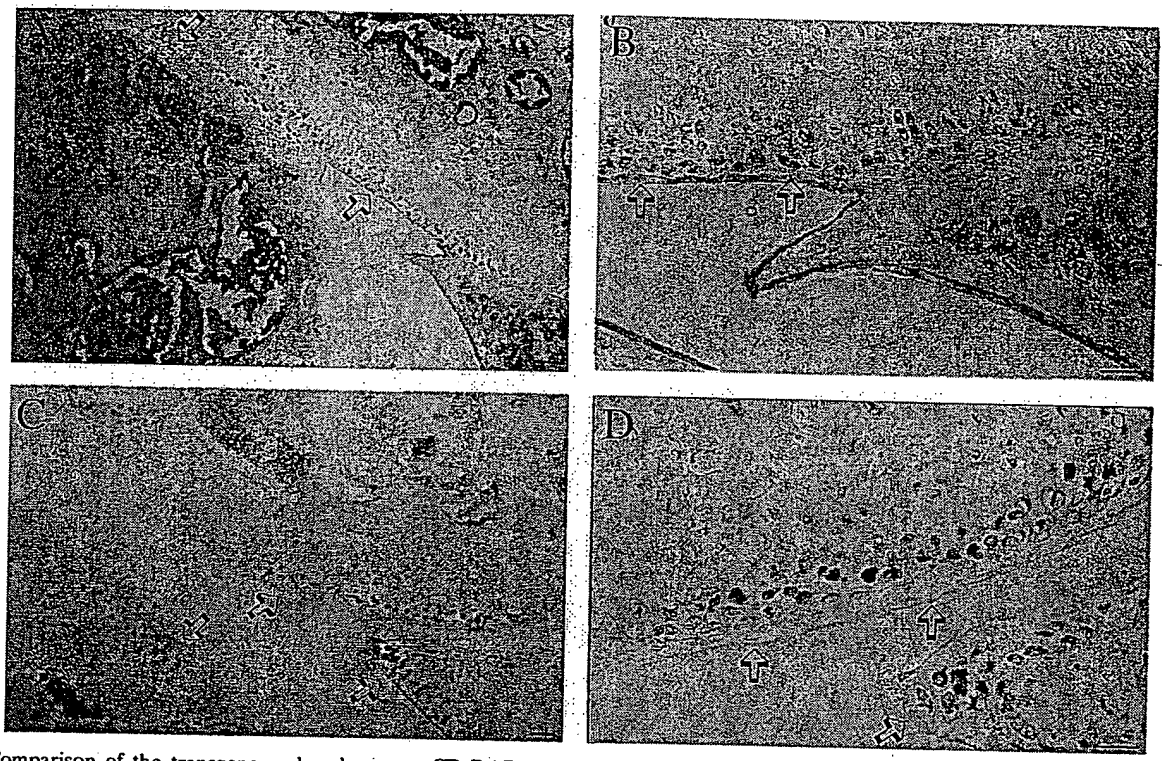

Fig. 5. Comparison of the transgene and endogenous CD-RAP gene expression in an 8-week knee joint tissues. (A and B) X-gal staining showed transgene generated *lacZ* expression (blue) at the articular cartilage (arrows) and meniscus. (C and D) Immunohistochemical analysis revealed that CD-RAP was localized in the articular cartilage and meniscus (arrows) as indicated by red color products. B and D are higher magnifications of A and C, respectively. Bars, 40 μm.

was the first cartilage gene shown to be regulated by AP-2 binding at 456 to 463 of the promoter (Xie et al., 1998). Sox9 contains a high mobility group (HMG)-type DNA binding domain and a transactivation domain within the 100 carboxy-terminal amino acids (Grosschedl et al., 1994; Südbeck et al., 1996). Sox9 is able to activate the expression of several cartilage genes, including collagen types II and XI (Bell et al., 1997; Bridgewater et al., 1998; Zhou et al., 1998; Lefebvre et al., 1998a) and we have recently demonstrated that Sox9 is capable of stimulating CD-RAP promoter activity through a Sox9 binding site at 404 to 410 (Xie et al., 1999). However, studies also suggest that other transcription factors are involved in the initiation of chondrogenesis as Sox9 is unable to activate cartilage-specific gene expression in non-chondrogenic cells (Lefebvre and de Crombrugghe, 1998b; Xie et al., 1999). De Crombrugghe and colleagues (Zhou et al., 1998) have shown that a 48-bp enhancer element in the first intron of type II collagen containing a Sox9 binding site is capable of directing chondrocyte-specific expression in transgenic mice. However, mutation in each of three HMG-like domains in this fragment (only one of which binds to Sox9 in vivo) abolished the cartilage-specific expression in vitro and in vivo (Zhou et al., 1998) implying that other HMG sequences are important for high levels of transcription. Similar HMG-type sites in type XI collagen were found to be essential for its enhancer activity and are able to generate specific transgene expression in some cartilaginous tissues (Bridgewater et al., 1998).

In these transgenic mice, all of the DNA constructs contain the AP-2 and Sox9 motifs, however, only the 2251 *lacZ* generated high levels of β-galactosidase activity in the correct temporal and spatial expression. Although caution must be exercised in the interpretation of negative expression in transgenic mice, the observation that both 2068 bp and 1427 bp of the promoter failed to generate *lacZ* expression at any tissue suggests that the segment upstream of 1427 of the mouse CD-RAP 5′-flanking sequence contains transcriptional regulatory elements essential for the induction of gene expression. Sequence analysis of DNA between 1427 and 2251 revealed additional HMG binding domains between 2251 and 2000 (Purple and Sandell, unpublished results). The lack of expression in any tissue may also suggest that the DNA between 1427 and 3 may contain suppressor sequences active in non-cartilage cells. Further studies will be performed to identify the motif(s) responsible for the induction of expression and to characterize the mechanism of gene activation and inhibition.

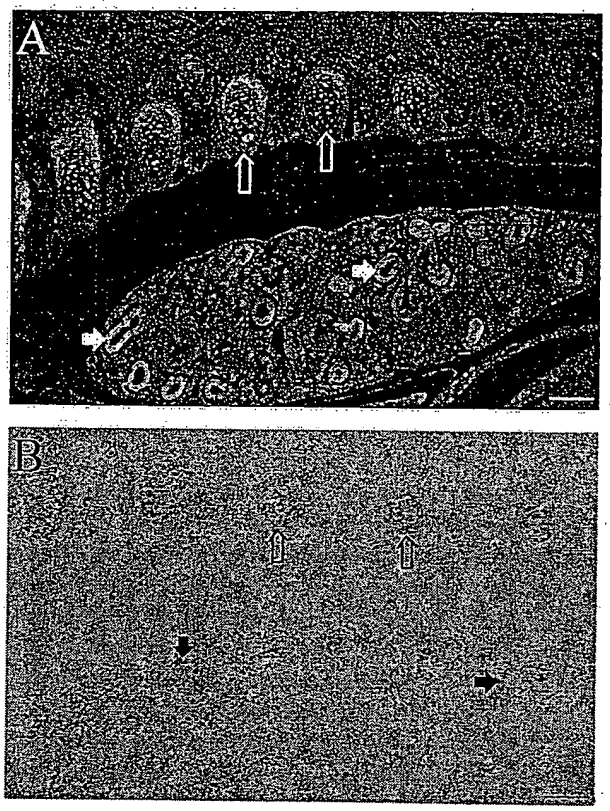

Fig. 6. Comparison of transgene 2251*lacZ* and endogenous CD-RAP gene expression in a 13.5-day embryo. (A) Darkfield image of transgene expression. Positive X-gal staining (pink, reflective) was observed in cartilaginous lung bronchi (filled arrow) and rib cartilage (empty arrow). (B) Immunostaining of CD-RAP (red) in cartilage of lung bronchi (filled arrow) and rib cartilage (empty arrow). Bars, 50 μm.

4. Experimental procedures

4.1. Construction of the mouse CD-RAP promoter-lacZ fusion gene

The expression vector used was placF, which contains the *lacZ* reporter gene, followed by a sequence of the murine protamine gene that supplies an intron and a polyadenylation signal (Mercer et al., 1991). Three different lengths of the mouse CD-RAP promoter were generated by PCR amplification using a 3′-antisense primer that bound at 3 relative to the CD-RAP translational start site in conjunction with a 5′-sense primer that bound at various distances within the CD-RAP upstream flanking sequences (for sequence, see Bosserhoff et al., 1997a). To facilitate subcloning of the amplified fragment, the sense primer contains a *Xba* I restriction site adaptor, and the antisense primer contains a *Sal* I recognition site. After digestion with *Xba* I and *Sal* I, 2251, 2068 and 1427 bp of the promoter fragments were cloned into the polylinker regions of placF by standard recombinant techniques to generate three DNA constructs (2251 *lacZ*, 2068 *lacZ*, and 1427 *lacZ*, respectively).

4.2. Generation and identification of transgenic mice

The DNA constructs were digested with *Xba* I and *Hind* III to release the inserts from their vector sequences. The enzymes cleaved upstream of the mouse CD-RAP promoter and downstream of the polyadenylation signal. The fragments containing the 2251 *lacZ*, 2068 *lacZ* and 1427 *lacZ* transgenes free of the vector sequences were isolated from agarose gel and the purified DNAs were microinjected into

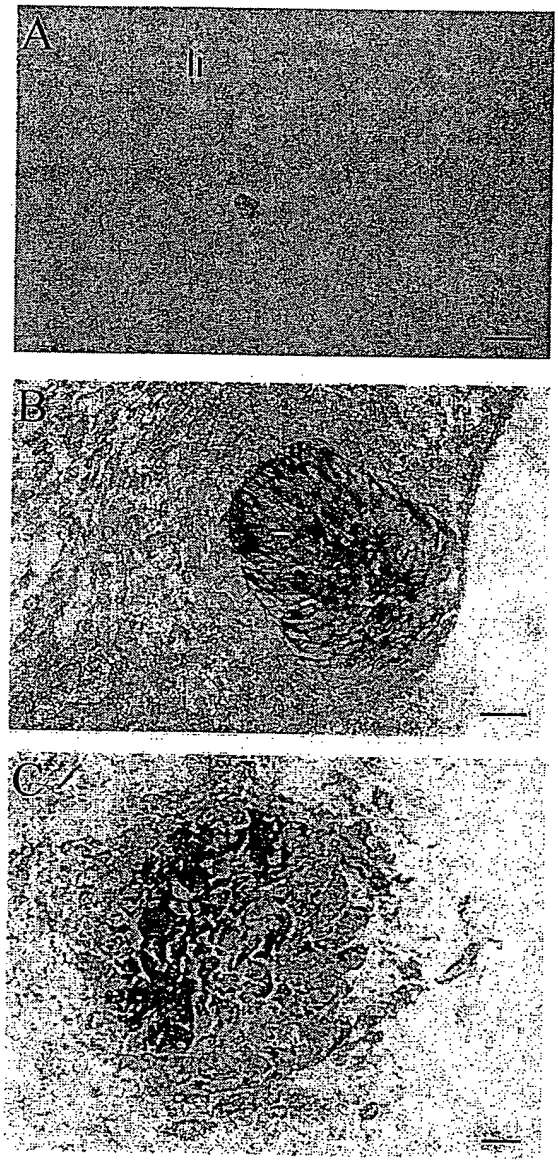

Fig. 7. Transgene expression in mammary buds. A 13.5-day p.c. embryo was stained with X-gal to define the mammary bud and then was sectioned on a microtome. (A) The blue color represents the X-gal reaction products in the mammary bud. No staining was detected in liver (li). (B) Higher magnification of mammary bud X-gal staining shown in A. (C) Immunolocalization of CD-RAP in mammary bud. Immunostaining is indicated by the red color products in the cytoplasm of epithelial cells. Bars, 200 μm (A); 20 μm (B,C).

the pronuclei of fertilized eggs from B6SJL hybrid to generate transgenic mice, as described previously (Hogan et al., 1994). The surviving eggs were implanted into pseudopregnant foster mothers. Founder mice were identified by PCR assays of the genomic DNA extracted from tail. The *lacZ*-specific primers used in PCR were as follows: sense, 5′-GCATCGAGCTGGGTAATAAGCGTTGGCAAT-3′; antisense, 5′-GACACCAGACCAACTGGTAATGGTAGCGAC-3′, which were expected to amplify an 822 bp fragment (Hanley and Merlie, 1991). Transgenic mouse lines were established and maintained by outbreeding with the B6SJL wild type mice. To analyze the temporal and spatial pattern of the β-galactosidase expression in the established lines, the positive founders or F1 males were mated with the B6SJL wild type females and embryos were isolated at different days of gestation. The day of the vaginal plug was designated day 0.5 of gestation.

4.3. LacZ staining and histochemistry

To assess the spatio-temporal expression pattern of transgenes, the β-galactosidase activity was detected in whole embryo as described (Bonnerot and Nicolas, 1993). Briefly, embryos were liberated from uterus and fixed in 4% paraformaldehyde at 4°C for 20–40 min on a rocker. After overnight incubation at 30°C in the staining solution with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma Chemical Co., St. Louis, MO, USA), the positive embryos were washed in phosphate-buffered saline, photographed, and post-fixed in 4% paraformaldehyde overnight. For histological examination, the stained embryos were dehydrated in ethanol and embedded in paraffin. Sections, 5–10-μm thick, were sliced on a microtome and counterstained with eosin.

4.4. Immunohistochemistry

To examine the endogenous CD-RAP expression, we carried out immunohistochemistry to localize CD-RAP. Antibodies against human recombinant CD-RAP MIA were generated in rabbits by Boehringer-Mannheim, Penzburg, Germany. Western blots and immunoprecipitation from melanoma cell lysates indicate that the antiserum was specific for CD-RAP (Bosserhoff et al., 1997a). The paraffin-embedded tissues were digested with 0.4% pepsin in 0.01 N HCl for 5–10 min at room temperature. The antibodies against CD-RAP were used at a dilution of 1:500. Preimmune serum was used as the control for the primary antibody. Immunohistochemistry was carried out using a DAKO LSAB 2-HRP Kit (Dako Corp., Carpinteria, CA, USA) according to manufacturer's instructions. For immunostaining in mammary buds, the X-gal staining was carried out overnight to identify the mammary gland before tissue sectioning.

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